# PHOSPHOFRUCTOKINASE AND THE REGULATION OF GLYCOLYSIS IN LYMPHOID TISSUE

*by*

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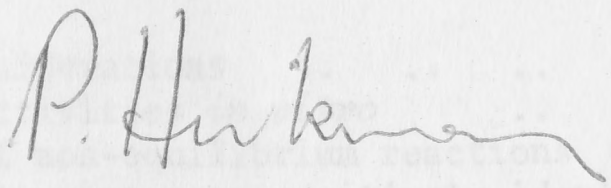
All the results contained in Chapter 3 on the purification and properties of pig spleen phosphofructokinase, were my own work. The results in Chapter 2 were obtained in collaborative projects with other workers in this laboratory. Freeze-clamping experiments and investigations of the Graft-versus-Host reaction in rat spleen were done in collaboration with Mr (now Dr) D. Suter, whilst the data on intracellular water spaces and the Con A stimulation of glycolysis in rat thymocyte was gathered by Mrs G. Keig, during the course of her B.Sc. Honours project.



P. HICKMAN

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## ABSTRACT

1. Profiles of the glycolytic intermediates were determined in freeze-clamped rat spleen and thymus. Hexokinase, phosphofructokinase and pyruvate kinase were all found to catalyze "non-equilibrium" reactions *in vivo* in both tissues, whilst the glucose carrier and aldolase were found to catalyze reactions intermediate between "equilibrium" and "non-equilibrium" position.

2. Under a variety of conditions where glycolytic flux rates were altered in both tissues, the glucose carrier/hexokinase and phosphofructokinase were both found to be regulatory.

3. The data presented on the regulation of glycolysis in lymphoid tissue indicates that two separate patterns of regulation may be distinguished:

   (i) *emergency glycolysis*, which occurs when either the tissue's respiratory system is perturbed or cytoplasmic phosphorylation potential is lowered; and

   (ii) *immunological glycolysis*, which occurs when the tissue is presented with an immunological challenge, by either mitogenic lectins or allogeneic lymphocytes. This regulatory pattern is characterized by elevated levels of the hexose-monophosphates.

4. Pig spleen phosphofructokinase has been purified 800-fold with a yield of 17%. Two isoenzymes were separated by DEAE-cellulose column chromatography. In common with the enzyme from other mammalian sources, pig spleen phosphofructokinase has a pH optimum of 8.2. It displays

sigmoidal kinetics with respect to F6P concentration, and its cooperative behaviour is very dependent upon pH, protein concentration and the concentration of MgATP.

5. The spleen enzyme was tested for its response to a wide variety of potential effector molecules, and it was found that possible *in vivo* effector molecules included ADP, AMP, F6P, FDP, aspartate and $PO_4^{2-}$ as activators, whilst ATP, citrate and $Mg^{2+}$ and $Ca^{2+}$ may be important inhibitors.

6. A series of experiments was conducted to investigate more fully the mechanism of action of some of the enzyme's effector molecules, and it was found that while AMP, ADP, $PO_4^{2-}$ and FDP activate phosphofructokinase and protect it from inactivation by MgATP, aspartate activates without protecting the enzyme, and $K^+$ and $NH_4^+$ are potent activators that appear to offer some protection, but only when F6P is present.

7. The *in vitro* properties of phosphofructokinase were compared with the data relating to the *in vivo* regulation of glycolysis. It is concluded that phosphofructokinase activity in lymphoid tissues *in vivo* correlates with, and is altered primarily by changes in the levels of intracellular $PO_4^{2-}$ and perhaps FDP.

8. A brief resumé of work done at the whole cell level is presented, in order to give a wider metabolic context to the experimental work contained within this thesis.

## PUBLICATIONS

1. Carbohydrate metabolism in rat lymphoid tissue, with special reference to the graft-versus-host reaction. Abstract. Suter, D.A.I., Hickman, P.E. and Weidemann, M.J. (1972) Proc. Aust. Biochem. Soc. 5, 16.

2. Modulation of phosphofructokinase activity by positive effectors. Hickman, P.E. and Weidemann, M.J. (1973) FEBS Letts. 38, 1.

3. Phosphofructokinase and the regulation of glycolysis in lymphoid tissue. Abstract. Hickman, P.E. and Weidemann, M.J. (1974) Proc. Aust. Biochem. Soc. 7, 40.

## ABBREVIATIONS

Apart from those listed below, abbreviations used are those accepted by the Biochemical Journal (The Biochemical Society, Instructions to Authors, 1973).

| | | |
|---|---|---|
| fructose-6-phosphate | ..... | F6P |
| fructose-diphosphate | ..... | FDP |
| glucose-6-phosphate | ..... | G6P |
| phospho-creatine | ..... | P-creatine |
| phosphoenolpyruvate | ..... | PEP |
| phosphoglycerate | ..... | PGA |
| *di*phosphoglycerate | ..... | diPGA |
| triose phosphate | ..... | triose-P |

# CHAPTER 1

## INTRODUCTION

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# INTRODUCTION

## 1.1 ELUCIDATION OF THE GLYCOLYTIC SEQUENCE

The term glycolysis was first used to describe the disappearance of carbohydrate during metabolic activity. Warburg later refined this definition to the splitting of carbohydrate into lactic acid (1). Elucidation of the complex enzymatic reactions involved in carbohydrate metabolism has led to a further restriction in definition, and the term is now used to denote "the stepwise degradation of glucose to pyruvate by way of fructose-diphosphate" (2).

Investigations into the structure and components of the glycolytic pathway arose from early experimental work on alcoholic fermentation in extracts of microorganismic cells. Harden and Young (3) observed that phosphate appeared to be essential for the operation of the pathway. They found that adding inorganic phosphate to a fermenting yeast juice containing glucose or fructose caused a large stimulation in fermentation rate. Careful measurement demonstrated that the increased quantities of $CO_2$ and alcohol produced were exactly equimolar with the phosphate utilized, implying that phosphate took a direct role in the pathway. They were able to demonstrate that added inorganic phosphate was bound to a hexose, and the phosphorylated compound was later identified as FDP. This phosphorylated sugar, when added to fermenting yeast juice, caused a large increase in fermentation rate, and from this result it appeared that the first intermediate of the glycolytic pathway had been identified.

The involvement of inorganic phosphate was further investigated by Robison (4) who demonstrated the presence of hexose-monophosphate in fermenting yeast-juice, and showed the molecular species involved to be a mixture of glucose- and fructose-6-phosphates. Later, in 1927, when

Meyerhof was able to demonstrate (5) a specific enzyme which had the capacity to phosphorylate glucose and other hexoses (hexokinase), the first section of the glycolytic pathway had been firmly established.

Identification of the intermediates between FDP and pyruvate was accomplished by a variety of methods. It was found, for example, that when iodoacetic acid was added to fermenting juice containing either glucose or FDP, although the added substrates disappeared, neither lactate nor ethanol were formed. Instead, an accumulation of triose-phosphates was observed. The triose-phosphates were shown to be glycolytic intermediates by demonstrating their utilization in, and stimulation of, the fermentation process.

Embden *et al* (6) found, whilst investigating the effect of ions on hexosephosphate synthesis, that in the presence of sodium lactate, fluoride or oxalate, abundant quantities of a compound, which they identified as phosphoglyceric acid, could be isolated as the barium salt. Careful measurement showed that for each mole of glyceraldehyde-3-phosphate converted to 3-phosphoglyceric acid, one mole of inorganic phosphate was consumed, and one mole of ATP was formed from ADP, implying the transient existence of a further intermediate. Negelein and Bromel (7) were able to isolate a compound which decomposed spontaneously in aqueous solution to 3-phosphoglyceric acid and which, by their criteria, was identical with 1,3-diphosphoglyceric acid.

3-phosphoglyceric acid can also be fermented to ethanol and $CO_2$ (6). When NaF was added to fermenting yeast juice containing 3-phosphoglyceric acid, the rate of fermentation fell, and concomitant with this, compounds later identified as 2-phosphoglyceric acid and phosphoenolpyruvate accumulated. With these demonstrations, the identity of all the intermediates of the glycolytic pathway had been established.

The Buchner's original term for their cell-free fermenting system was "zymase", and it was realised that the interconversion of the intermediates of the glycolytic system was dependent on a heat-labile component of the "zymase", i.e. the catalytic proteins, enzymes. However, as early as 1906, Harden and Young (3) were able to demonstrate that the function of the pathway was also dependent on a heat-stable, dialyzable fraction which they called "co-zymase". Extensive research has demonstrated that "co-zymase" is in reality a complex mixture of chemicals, and Krebs (8) lists 8 co-factors required before fermentation can occur.

Although the identification and sequencing of the glycolytic intermediates began very shortly after the Buchners' demonstration of cell-free fermentation, the identification, extraction and purification of the individual enzymes responsible for these interconversions, did not commence until 1927, when Meyerhof (5) reported the isolation of hexokinase. Much of the work on identification and characterization of the individual enzymes was done in Meyerhof's laboratory during the 1930's (e.g. 9, 10). However it was not until the mid-1960's that the most labile of these enzymes, phosphofructokinase, was purified to homogeneity in stable form (11). More will be said later of those enzymes involved in the regulation of glycolysis.

Clearly the work above describes a means by which the sequential fragmentation of sugars can occur. It does not, however, indicate any means by which the rate of this process can be controlled. Fletcher and Hopkins (12) noted as early as 1907 that glycolysis proceeds faster in working than in resting muscle. Since this early discovery, the work of numerous investigators has demonstrated that the rate of glycolysis can be varied by subjecting the experimental organism or organ to a variety of conditions, and that these changes in flux rate are controlled at

specific enzyme loci. In recent years a systematic procedure for identifying regulatory enzymes has been developed. The next section will discuss the essential features of this procedure.

## 1.2 SYSTEMATIC IDENTIFICATION OF REGULATORY STEPS

In 1967, Newsholme and Gevers (13) presented a theory of metabolic control, referring specifically to regulation of the interrelated pathways of glycolysis and gluconeogenesis in liver and kidney cortex. In essence, the principles underlying their approach, as applied to the glycolytic pathway alone, may be formulated in the following terms. Glycolytic flux is controlled by certain specific enzymes — regulatory enzymes.

(i) Those enzymes which are of major importance in regulating glycolytic flux are identified by specific criteria.

(ii) The properties of these regulatory enzymes are investigated in detail; this requires purifying the enzymes at least to the stage where metabolites and other enzymes which may obscure or interfere with the assay are removed.

(iii) On the basis of the properties observed, a theory for the control of glycolysis is proposed; and

(iv) The theory, or predictions which arise from it, are tested and the theory is modified or expanded accordingly.

Sections of this approach have been further elaborated by Newsholme (14) and Rolleston (15).

Although a large number of criteria have been employed for identifying regulatory enzymes, only four are considered here.

### 1.2.1 Teleological considerations

The living cell performs a variety of metabolic functions and in the interests of its own viability may be expected to develop mechanisms whereby this task is accomplished most efficiently i.e. with the least expenditure of energy. For this reason, one might predict that a regulatory site will occur early in the pathway (to minimize uncontrolled metabolism) and/or at a site which is either unique for the pathway, or has minimal overlap with other pathways (so that regulation can be specific). This will be considered as it actually applies to the glycolytic pathway later.

### 1.2.2 Maximal enzyme activities *in vitro*

Flux through a metabolic pathway can proceed only as fast as the slowest reaction in its sequence. For this reason, one might expect an enzyme with regulatory capability to have a lower maximal catalytic capacity than other enzymes of the same pathway. Hence it is of value to determine the maximal catalytic activity of each enzyme in the pathway. Unfortunately, measurements must be made on tissue extracts, where the procedures involved in disrupting the tissue and extracting the enzymes, may cause unknown and uncontrollable losses in activity. However, most non-regulatory enzymes have catalytic capacities ten to one hundred times greater than their regulatory counterparts, so that it may be considered unlikely that enzyme loss during extraction would be sufficient to disguise this difference.

### 1.2.3 Identification of "non-equilibrium" reactions

Krebs stated in 1946 (16), that freely reversible reactions are unlikely to be of importance in controlling metabolism, as the enzymes responsible will be able to freely interconvert their substrates so that the reactions involved remain near their equilibrium positions. Only when an enzyme's activity falls below that of the preceding enzymes of

the pathway, causing an accumulation of its substrate, is the enzyme able to exert the rate-limiting capacity necessary for regulation. This will result in a marked deviation of the reaction from its equilibrium position, and the reaction will become non-equilibrium. Non-equilibrium reactions may be identified by determining the concentration of substrates and products for each reaction in freeze-clamped tissue, and subsequently determining the mass-action ratio. Comparison of the calculated mass-action ratio with the apparent equilibrium constant indicates whether the particular enzyme is catalyzing an equilibrium or non-equilibrium reaction. It must be pointed out that regulation *can* occur at equilibrium steps, but this is of the "open loop" type, where a substrate or cofactor of one of the reactions of a reversible enzyme may become limited — e.g. pyridine nucleotide regulation of glyceraldehyde-3 phosphate dehydrogenase. Non-equilibrium reactions, which proceed with large changes in free energy are, in practice at least, irreversible.

### 1.2.4 Use of altered flux rates to positively identify regulatory enzymes

Rolleston and Newsholme (17) defined a regulatory enzyme as one "whose activity controls the rate of flux through a metabolic pathway (i.e. a non-equilibrium reaction) and whose activity is regulated by factors other than the substrate concentration".

To determine which non-equilibrium enzymes are regulatory, is best done by adoption of a principle suggested by Krebs (18). That is, if the substrate concentration of a non-equilibrium enzyme changes in the *opposite* direction to a change in flux through the pathway, then that enzyme is considered to be regulatory under the conditions which induced the flux change. This identification is only a positive one however; failure to comply with this criterion does not constitute evidence that the enzyme is *non*-regulatory.

Clearly it is necessary to know the *in vivo* levels of substrates and cofactors for the different enzymes, in order to determine regulatory sites by the above criteria. This can be accomplished by crushing the whole organ between aluminium tongs cooled to -180°C in liquid nitrogen (19). This very rapidly lowers the intracellular temperature to a level where significant metabolic activity can no longer occur. Glycolytic intermediates can then be extracted by perchloric acid treatment, and their concentrations determined by assay.

There is a major problem associated with this technique. Freeze-clamped values indicate the total amount of a particular intermediate, but give no indication of its intracellular distribution in relation to the enzyme for which it is a substrate. This problem is of particular importance when an enzyme is located within an organelle whose membranes are not freely permeable to the reactants and products of that enzyme. Suter (20) has recently discussed this problem in relation to the pyruvate dehydrogenase reaction. Fortunately, in investigations of glycolysis, compartmentation is not a major problem, as both the enzymes and intermediates are located predominantly in one compartment, the cytoplasm. The problem of compartmentation of glucose between the intracellular and extracellular spaces will be discussed in the experimental section of this thesis.

## 1.3 EXPERIMENTAL DETERMINATION OF REGULATORY LOCI

In this section, the four criteria that were discussed in Section 1.2 above, will be considered in the light of the experimental evidence available.

### 1.3.1 Teleological considerations

On a teleological basis, both hexokinase and phosphofructokinase might be expected to catalyze regulated reactions. Hexokinase catalyzes

the first reaction involved in the metabolism of glucose, and phosphofructokinase catalyzes the first reaction which uses a substrate unique to glycolysis and which is not involved in branch points to any other pathway.

Although not strictly part of glycolysis, the transport of glucose into the cell is a logical site for regulation, and rate limitation at this point would clearly have important consequences for carbon flux through the pathway.

### 1.3.2 Maximal enzyme activities *in vitro*

Maximal *in vitro* glycolytic enzyme activities have been measured in a number of rat and mouse tissues (e.g. 21, 22), and in several human tissues, both normal and malignant (e.g. 23, 24). In all cases the same basic pattern has been observed: i.e.,

(i) all enzymes have catalytic capacities many times greater than the overall flux rates estimated;

(ii) the *ratios* of the enzyme activities along the pathway are strikingly similar from tissue to tissue; and

(iii) there is always one small group of enzymes, whose activities are consistently lower than those of other members of the pathway; members of this group are hexokinase, phosphofructokinase, aldolase and, perhaps, enolase.

### 1.3.3 Identification of non-equilibrium reactions

Comparison of the mass-action ratios observed in liver (25), perfused rat heart (26), rat brain (27) and Ehrlich ascites tumour cells (28) with the apparent equilibrium constants for the different enzymes, show that hexokinase, phosphofructokinase and pyruvate kinase are all far removed from equilibrium. In addition, in rat brain and heart,

glyceraldehyde-3-phosphate dehydrogenase appears to catalyze a non-equilibrium reaction.

### 1.3.4 Use of altered flux rates to positively identify regulatory enzymes

From the data considered to date, both hexokinase and phosphofructokinase fit the criteria for a potentially regulatory enzyme in every way, i.e. they are at logical locations for regulation, they have comparatively low activities *in vitro*, and both catalyze irreversible non-equilibrium reactions. In contrast, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase are comparatively high activity enzymes. Glyceraldehyde-3-phosphate dehydrogenase catalyzes a reversible reaction and appears to be regulatory only if the level of $NAD^+$ or NADH drops to a level low enough to limit catalytic activity to one particular direction. The only obvious reason for pyruvate kinase to be regulatory is that it catalyzes the last reaction before the pathway splits into oxidative and anaerobic branches. It does catalyze a virtually irreversible reaction, with an apparent equilibrium constant (of the order of $10^4$) that is very much in favour of the products.

To finally determine which non-equilibrium enzymes are regulatory, it is necessary to alter the rate of flux through the pathway, and to see which enzymes show the changes in substrate concentration, characteristic of a regulatory role (see 1.2.4). Examples of experiments conducted to determine the regulatory loci of glycolysis are given below.

When muscle is stimulated repetitively, flow of carbon through the glycolytic system may increase by several hundred fold. Karpatkin *et al* (29), using electrical stimulus of frog sartorius muscle as their model, were able to show by determination of glycolytic intermediate concentrations, that the large and rapid increase in glycolytic rate from glycogen was accompanied by a correspondingly large and rapid activation

of both phosphorylase and phosphofructokinase. Furthermore, when exogenous glucose was the substrate, the increased flux rate was accompanied by an activation of hexokinase. During electrical stimulation, the intracellular pH rises initially, apparently due to the hydrolysis of phosphocreatine (30). Hexokinase and phosphofructokinase are known to be more active at pH values higher than 7.0, so the pH change alone may be sufficient to account for part of the observed stimulation. Additionally, the concentrations of several of the effector molecules which regulate phosphofructokinase activity *in vitro* change during stimulation, and the direction of change is consistent with a sustained activation of this enzyme (31). The complex processes associated with phosphofructokinase activation will be discussed later.

Perfusion of isolated organs of rodents and other small mammals has become extremely popular as a means of investigating metabolic regulation in functioning tissues. It has considerable advantage over other *in vitro* tissue preparations such as slices and homogenates, because less damage is caused to the organ during its preparation, and because the supply of oxygen and substrates can be more precisely varied and controlled.

Newsholme and Randle (32) have looked at glycolytic regulation in the perfused rat heart. Perfusion was terminated by freezing, and the levels of the various hexosemonophosphates measured. Making the tissue anoxic was found to increase the activities of phosphofructokinase and hexokinase, both in the presence and absence of insulin. Because the phosphohexose-isomerase reaction is reversible, one effect of stimulating phosphofructokinase will be to lower G6P levels. G6P inhibits hexokinase (33), and it was suggested that the observed stimulation of hexokinase was due to the removal of this constraint and was thus secondary to the stimulation of phosphofructokinase. Regen *et al* (34), and Hems and Gaja

(35) have also shown that anoxia stimulates phosphofructokinase in perfused rat heart and rat kidney.

Weidemann *et al* (36) looked at the effects of added adenine nucleotides on glycolysis and gluconeogenesis in the perfused rat kidney. In their system, part of any ATP added to the perfusate was rapidly converted to AMP, but further hydrolysis was prevented by ATP- and $PO_4^{2-}$-mediated inhibition of the AMP-degrading enzymes (37). They found that addition of ATP together with glucose caused an increase in the concentration of glycolytic intermediates between FDP and pyruvate. From this data they concluded that the AMP formed was stimulating phosphofructokinase and inhibiting fructose-diphosphatase (see Section 1.4.5 below).

The experimental work described above is intended only to provide selected examples of the methods available for determining the regulatory loci of glycolysis. Clearly, the most common sites of regulation are found at hexokinase and phosphofructokinase. This section will close by briefly considering some of the information available on the properties of enzymes located at the major regulatory sites of glycolysis.

### 1.3.5 The regulatory loci of glycolysis

A regulatory enzyme was defined earlier, as one which controls the rate of flux through a pathway, and whose activity is regulated by factors other than the concentration of its substrate.

Although not strictly an enzyme of the glycolytic pathway, the possible rate-limiting role of the glucose carrier must be considered in any discussion of glycolytic regulation. Transport of exogenous glucose into the cell is obviously necessary for the pathway to function in the absence of unlimited supplies of stored glycogen, and in rat cardiac muscle (38), diaphragm (39) and adipose tissue (40), it is a major rate-limiting step involved in glucose utilization. In these tissues,

entry of glucose is greatly accelerated by insulin and, as described above, anoxia also appears to stimulate transport.

Mammalian hexokinase has a high sensitivity to inhibition by its product, G6P. This inhibition may be overcome by $PO_4^{2-}$. In addition, MgADP is an inhibitor, acting apparently in competition with the other substrate, MgATP. The evidence available suggests that G6P and $PO_4^{2-}$ bind at a separate "allosteric" site (2).

The evidence available implicating phosphofructokinase as a regulatory enzyme is much greater. In all mammalian tissues where glycolytic flux is altered, rapid changes in phosphofructokinase activity are usually found to be principally responsible. In the experimental section of this thesis, phosphofructokinase is shown to be a major regulator of glycolytic flux in rat spleen and thymus and, additionally, a purification and detailed investigation of the kinetic properties of the enzyme from pig spleen has been undertaken. For this reason, the known properties of phosphofructokinase will be reviewed in some detail, concentrating mainly on the information available up to the commencement of work on this project (in March 1971).

## 1.4 PHOSPHOFRUCTOKINASE

### 1.4.1 Introduction

The hexose-phosphates, which are the substrate and product of the phosphofructokinase reaction, were identified during early investigations into the sequence of the glycolytic pathway. It was not until 1936, however, that a specific enzyme was shown to be responsible for this reaction (41). The first suggestion that phosphofructokinase may play a significant role in regulating glycolysis came in 1942, when Cori (42) reported that under certain circumstances, hexosemonophosphates may accumulate in muscle extracts, without any corresponding increase in the

amount of lactic acid formed. In 1951, Muntz and Hurwitz (43) demonstrated that rat brain phosphofructokinase is greatly stimulated by $NH_4^+$. Since this time, phosphofructokinase has been positively identified as the principal regulatory enzyme of glycolysis in every major mammalian tissue studied. It has been purified from a number of sources and its properties exhaustively examined. In addition to investigations of the allosteric regulatory properties of phosphofructokinase *in vitro*, a large body of work has been devoted to examining the physical structure of the enzyme and, more recently, to the role different isoenzymes may play in fulfilling the metabolic requirements of different tissues. All of these aspects will be considered in detail below.

1.4.2 The purification of phosphofructokinase from different tissues

Mammalian phosphofructokinase is very labile. The first reported purifications were by Taylor in 1951 (44), from skeletal muscle, and by Muntz in 1953 (45) from dog brain. Taylor achieved a 30-fold purification by repeated $(NH_4)_2SO_4$ precipitation whilst Muntz, although achieving only a small degree of purification, was able to markedly increase the stability over that obtained in crude homogenates.

The relative amounts of phosphofructokinase in different tissues varies considerably. For example, Shonk and Boxer (22) reported that skeletal muscle, on the basis of units of enzyme per g. wet wt. tissue, has twice as much phosphofructokinase as brain, four times as much as cardiac muscle and sixteen times as much as liver. When considered in terms of specific activity, i.e. in units of enzyme/mg. protein, the difference is even greater, as liver, for example, contains more protein per g. tissue than does muscle. For these reasons, most of the initial attempts to purify phosphofructokinase were made using skeletal muscle from various animals.

Although several laboratories worked on the purification of skeletal muscle phosphofructokinase during the 1950's and early 1960's (e.g. 46, 47, 48), it was not until 1965 that both homogeneous *and* stable preparations of the enzyme were reported. Almost simultaneously, Ling *et al* (11), Uyeda and Racker (49), and Parmeggiani *et al* (50) reported the purification of the skeletal muscle enzyme to homogeneity, whilst Mansour *et al* (51) reported a similar result for the heart enzyme.

Highly biosynthetic tissues such as liver and kidney cortex, which also possess a gluconeogenic capacity, are unlikely to have the same energy requirements from glycolysis as muscle. For this reason it was considered that the enzyme from these sources might have kinetic properties significantly different to those of the muscle enzyme. Although phosphofructokinase is present in much smaller quantities in tissues other than muscle, purifications from a number of other tissues have been made.

The first substantial purification from liver was made by Underwood and Newsholme (52), who in 1965 reported a 35-fold increase in the specific activity of the rat liver enzyme. More recently, Brock (53) and Kemp (54) have reported 500- and 2500-fold purifications of the sheep and rabbit liver enzymes respectively.

In 1969, it was reported that a 50% decrease in phosphofructokinase content led to a significantly reduced erythrocyte survival (55). This decrease is apparently hereditary, and was classified as a Type VII glycogen storage disease. Interest in the disease led to attempts to purify the erythrocyte enzyme, and in 1969 Layzer *et al* (56) reported the partial purification of human erythrocyte phosphofructokinase. Finally, in 1972, Tarui *et al* (57) purified the rabbit erythrocyte enzyme to homogeneity, giving it a specific activity similar to that of the homogeneous muscle enzyme, i.e. 140 U/mg. protein.

Investigations into the purification and properties of the brain enzyme have been conducted largely by Lowry and Passoneau. In 1966, they reported (58) a 240-fold purification of the enzyme from sheep brain with a final specific activity of 18 U/mg. protein. Krzanowski and Matchinsky (59) have partially purified phosphofructokinase from rat brain, but only to a specific activity of approximately 1 U/mg. protein.

These represent the major tissues from which purifications have been attempted. Other purifications have been made of the enzyme from kidney cortex (60), sperm (61), jejunal mucosa (62), thymus (63), and hepatoma (64).

### 1.4.3 The molecular structure of phosphofructokinase

It was not until homogeneous preparations of phosphofructokinase were available that attempts were made to investigate the physical structure of the enzyme. What was most obvious to workers at the outset, was the ability of the enzyme to exist in forms of several different molecular weights (11, 50, 65, 66). Paetkau and Lardy (67) and Mansour and Ahlfors (65) were able to show that the smallest active form of the enzyme had a molecular weight of approximately 380,000 daltons.

Paetkau *et al* (68) and Paetkau and Lardy (67) investigated the subunit structure of the skeletal muscle enzyme in detail. Using ultracentrifugation techniques allied to urea and SDS treatments, they found forms of phosphofructokinase with molecular weights 93,000, 192,000 and higher multiples. From amino acid analyses, they concluded that the species with molecular weight 93,000 constituted the basic unit or protomer* of phosphofructokinase. In support of this, Kemp and Krebs (69) found 90,000 daltons to be the binding unit molecular weight for F6P, AMP and ADP, although this unit could bind three molecules of ATP.

*"The identical subunits associated within an oligomeric protein are designated as protomers" (Changeux, 72).

Paetkau *et al* (68) found additionally that treatment of the 93,000 dalton molecular species with 6M guanidinium chloride generated species with molecular weights of about 24,000. These however were of more than one kind, as shown by paper chromatography of tryptic digests.

Considering their results alongside those of Kemp and Krebs, Paetkau *et al* concluded that each protomer consisted of one catalytic and three regulatory subunits. Mansour and coworkers (70, 71) have also examined the binding properties of phosphofructokinase. They found that each protomer of enzyme bound two molecules of F6P, four molecules of ATP and two molecules of citrate. From these results, they proposed that each protomer consists of two catalytic and two regulatory subunits.

More recently, Lardy's laboratory has reported (73) that they are unable to generate species with molecular weights less than 50,000 by any means. They suggested that the guanidine used in the earlier studies may have degraded the enzyme. Although this now throws doubt on the structure of phosphofructokinase as proposed by Mansour (103, i.e. association/dissociation between dimer and tetramer) subsequent data will still be discussed in these terms, although it will be implicit in this discussion that where "active tetramer" is referred to, "active hexamer" may be the correct form. Concomitantly with this, there may be a need to accept slightly different molecular weights for the various species.

It appears that the basic active unit of phosphofructokinase, *in vivo*, is the 360,000 dalton tetramer. At approximately pH 7.0 or below, the enzyme may reversibly dissociate into inactive dimers of molecular weight 180,000 daltons. The inactivation process is enhanced by ATP and other inhibitors, and blocked by activators such as AMP and FDP. It is not known if the higher molecular weight forms seen in the ultracentrifuge exist *in vivo*. The biological importance of the interconversion between dimeric and tetrameric species, and the mechanism involved, will be

discussed in Section 1.4.5(d) below. Figure 1-1 summarizes the data available on the different molecular-weight forms of phosphofructokinase.

### 1.4.4 Isoenzymes of phosphofructokinase

The first demonstration that mammalian phosphofructokinases from different tissues had different kinetic properties was made in 1964 by Lowry and Passoneau (74). They found that the response of the enzyme to several of its effectors in crude extracts of several rat tissues was quantitatively different.

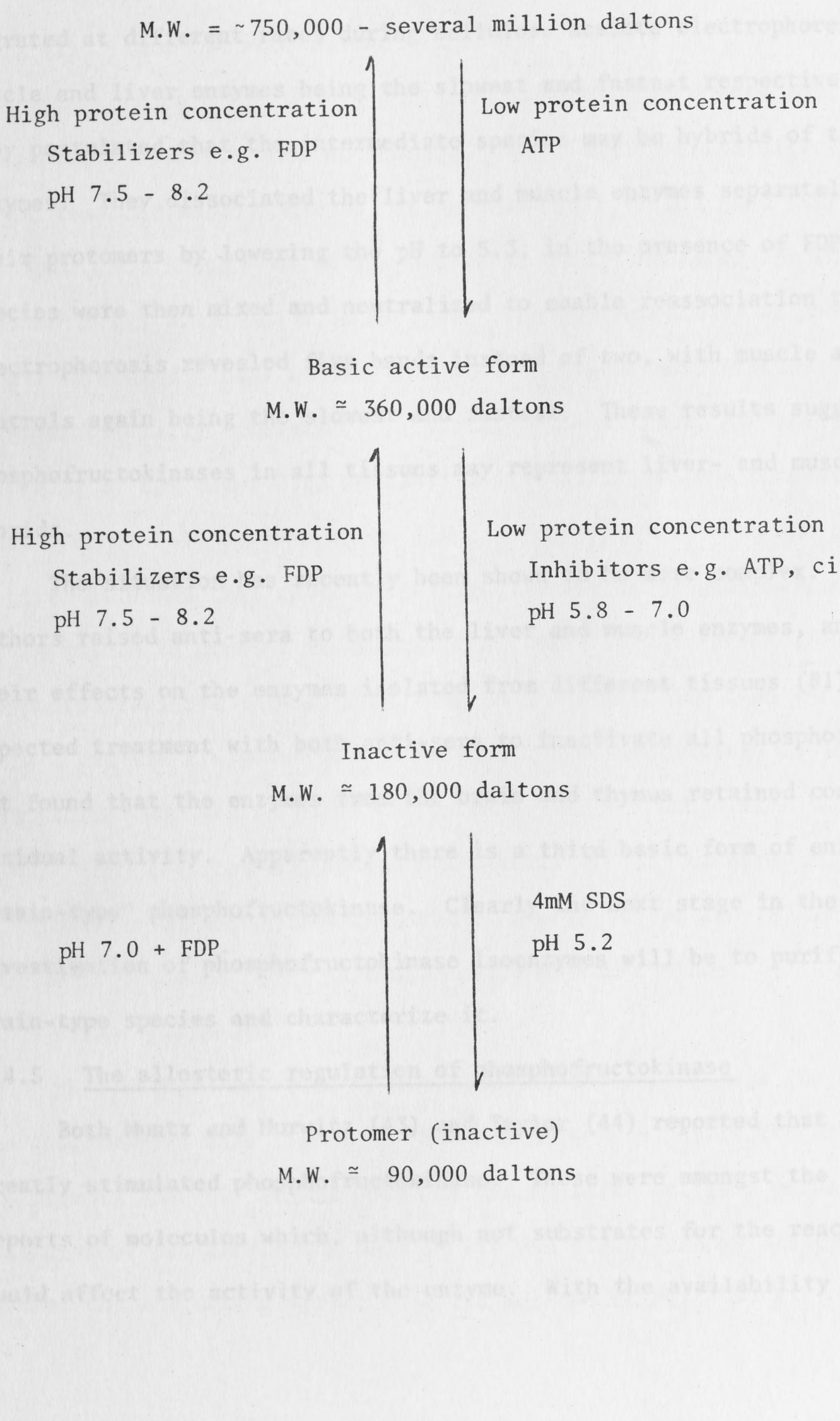
Further interest in the isoenzymes of phosphofructokinase was aroused with the discovery of a glycogen storage disease characterized by a deficiency of the enzyme in some tissues only. Sufferers of this disease had no enzyme activity in their muscles, only half normal activity in their erythrocytes, and essentially normal activity in their white blood cells (75, 76). Because of the selective involvement of certain tissues, Layzer and coworkers suspected the occurrence of isoenzymes. They examined the enzyme from several human tissues and, on the basis of kinetic, chromatographic and immunological methods, were able to demonstrate the existence of at least four different isoenzymes (56, 77).

Taylor and Bew (78) reported at about the same time, that rat tissues appeared to contain two different types of phosphofructokinase, a muscle-type and a liver-type. Enzyme extracts from a number of other tissues appeared to contain a mixture of these two main enzymatic forms.

Kemp (54) has made a comparison of certain of the kinetic properties of the liver and skeletal muscle enzymes. The muscle enzyme is more sensitive to activation by the adenine nucleotides and to inhibition by citrate and 3-PGA, whilst the liver enzyme is more sensitive to inhibition by ATP and 2,3-diPGA. *In vivo* concentration of ATP are of the order of 5.0 mM in muscle (79) and 2.7 mM in liver (25). Kemp suggested that the differing sensitivities of the two enzymes to ATP

Figure 1-1 The structure of mammalian phosphofructokinase

The information in this figure is for work done on either the heart or skeletal muscle enzyme, and is drawn from the following references: 65, 68, 69, 70, 71, 80, 103. Although the generation of sub-protomeric species has been reported (68), this data has since been retracted (73).



should be considered in the light of the concentration of ATP within the two tissues. Additionally he suggested that the muscle enzyme's greater sensitivity to adenine nucleotide activation was logical, as the muscle may at times, have to produce energy under partially anaerobic conditions.

Tsai and Kemp had observed that different phosphofructokinases migrated at different rates during cellulose acetate electrophoresis, with muscle and liver enzymes being the slowest and fastest respectively (80). They postulated that the intermediate species may be hybrids of these two enzymes. They dissociated the liver and muscle enzymes separately into their protomers by lowering the pH to 5.3, in the presence of FDP. These species were then mixed and neutralized to enable reassociation to occur. Electrophoresis revealed five bands instead of two, with muscle and liver controls again being the slowest and fastest. These results suggest that phosphofructokinases in all tissues may represent liver- and muscle-type hybrids.

The situation has recently been shown to be more complex. The same authors raised anti-sera to both the liver and muscle enzymes, and tested their effects on the enzymes isolated from different tissues (81). They expected treatment with both anti-sera to inactivate all phosphofructokinases, but found that the enzymes from the brain and thymus retained considerable residual activity. Apparently there is a third basic form of enzyme, "brain-type" phosphofructokinase. Clearly the next stage in the investigation of phosphofructokinase isoenzymes will be to purify the brain-type species and characterize it.

### 1.4.5 The allosteric regulation of phosphofructokinase

Both Muntz and Hurwitz (43) and Taylor (44) reported that $NH_4^+$ greatly stimulated phosphofructokinase. These were amongst the first reports of molecules which, although not substrates for the reaction, would affect the activity of the enzyme. With the availability of highly

purified and stable phosphofructokinase, large scale investigations into the kinetic properties of the enzyme from various sources have been conducted. Table 1-1 lists the major reported allosteric effectors of phosphofructokinase.

(a) Activators

(i) Adenine nucleotides

The adenine nucleotides, AMP, cAMP and ADP, are potent activators of phosphofructokinase. They do however, have a differential effect on the enzyme from different sources. In muscle, the $K_A$'s for AMP, cAMP and ADP are 0.035, 0.015 and 0.04 mM respectively, whilst in liver the comparable $K_A$'s are 0.21, 0.075 and 0.31 mM (54). The concentrations of AMP in both normal liver and normal muscle, are such that phosphofructokinase will be at least partially activated. In normal aerobic liver, AMP levels are typically of the order 0.25 mM (25), whilst in muscle the concentration is usually about half of this value (calculated from 79). The AMP concentration in liver is below that which half-maximally activates phosphofructokinase, but in muscle is present at several times this concentration (54). Liver phosphofructokinase is more sensitive to ATP inhibition than the muscle enzyme, and the closeness of the *in vivo* AMP concentrations to the $K_A$, suggest that AMP may be important in relieving ATP inhibition in this organ. Kemp (54) has suggested that AMP and ADP may be more important as regulators of phosphofructokinase in muscle, where energy may at times have to be produced under partially anaerobic conditions.

(ii) Phosphate

Phosphate is reported to activate phosphofructokinase (56, 58, 82). The true role of this ligand is somewhat uncertain, as in the earlier experiments (56, 58) it was added as the $K^+$ salt. $K^+$ is a potent activator of phosphofructokinase in its own right, and it was uncertain whether the activating effect observed was due to $PO_4^{2-}$ or $K^+$. However,

Table 1-1

The major allosteric effectors of mammalian phosphofructokinase

| ACTIVATORS | REFERENCE | INHIBITORS | REFERENCE |
|---|---|---|---|
| AMP | 52, 54 | ATP or MgATP | 52, 54, 58, 89 |
| cAMP | 54, 65 | Citrate | 53, 90, 91, 92 |
| ADP | 54, 58 | PEP | 49, 59 |
| $PO_4^{2-}$ | 56, 58, 82 | P-creatine | 49, 59 |
| FDP | 52, 58, 87 | 3-PGA | 54, 59 |
| $NH_4^+$ | 43, 83, 92 | 2-PGA | 54, 59 |
| $K^+$ | 54, 58, 83 | 2,3-diPGA | 54, 59 |
| F6P | 53, 58, 60, 88 | | |

Tejwani and Ramaiah (82) used the $Na^+$ salt and got essentially similar results. The $K_A$ is reported to be of the order 4 - 5 mM.

(iii) $NH_4^+$ and $K^+$

$NH_4^+$ and $K^+$ greatly stimulate phosphofructokinase from all mammalian tissues studied (54, 58, 83). The activation constants for each effector seem to be identical for both major isoenzymes, the $K_A$ for $K^+$ being approximately 17 mM, and for $NH_4^+$ approximately 0.35 mM (54). Intracellular concentrations of $K^+$ in various mammalian tissues vary between 60 and 100 mM (84), so that under normal *in vivo* conditions, phosphofructokinase will be fully activated with respect to $K^+$. Several authors have suggested that $NH_4^+$ may be an important regulator of phosphofructokinase activity (e.g. 20, 83, 85). However, pyruvate kinase is activated by both $NH_4^+$ and $K^+$, and it is thought that they share the same binding site (86). If, in like manner, they should share the same binding site on phosphofructokinase, then it is unlikely that $NH_4^+$ would be a physiologically important regulator.

(iv) FDP

FDP is perhaps the most potent activator of mammalian phosphofructokinase. Lowry and Passoneau (58) reported that the brain enzyme was half-maximally activated by only 1 μM FDP. The enzymes from other tissues, including liver (52, 87), heart muscle (65) and kidney cortex (60) are also readily activated by FDP.

(v) F6P

Although F6P is a substrate, and apparently does not have a separate allosteric binding site (70), it must be considered to have activating or at least deinhibiting properties. It has been reported in many papers (56, 58, 59, 60, 88) that increasing the F6P concentration substantially reduces the sensitivity of the enzyme to inhibition by ATP, citrate, PEP, P-creatine and the phosphoglycerates.

These molecules constitute the major known activators of phosphofructokinase. Their mechanism of action and suggested role in

regulating enzyme activity *in vivo* will be discussed later.

(b) Inhibitors

As a preface to this section, it is important to note that the degree of inhibition observed with any given compound is strongly influenced by pH, enzyme concentration, and the presence or absence of other effector molecules, either positive or negative. For this reason, quantitative values for inhibitor constants are perhaps not very meaningful, as different workers may have used significantly different conditions of assay.

(i) ATP

The first observation that ATP inhibited phosphofructokinase was made by Lardy and Parks (89) in 1956. They were uncertain of the mechanism involved and thought that it might act simply by chelating the essential divalent metal ion, $Mg^{2+}$. More recently it has been demonstrated that both ATP and MgATP inhibit phosphofructokinase (58, 67). Most studies on the regulatory properties of phosphofructokinase from mammalian tissues have reported ATP inhibition of the enzyme. Kemp (54) has observed differing sensitivities to MgATP inhibition (in his studies, $Mg^{2+}$ was always at least 2 mM in excess of the ATP concentration) between the liver and muscle enzymes. He suggested that the greater sensitivity of the liver enzyme to ATP may be related to the lower concentration of ATP found in liver. To avoid subsequent confusion, it must be emphasized here that only MgATP will serve as a substrate for phosphofructokinase, whilst both MgATP and ATP will act as inhibitors.

(ii) Citrate

Citrate was identified as an inhibitor of phosphofructokinase almost simultaneously in 3 separate laboratories (90, 91, 92). Both Garland *et al* (90) and Parmeggiani and Bowman (91) discovered the effect of citrate during investigations into the regulation of glycolysis in the diabetic rat heart. They observed an inhibition of glycolysis at the locus of phosphofructokinase, which was not accompanied by significant changes in

the effector molecules known at that time, i.e. no increase in ATP or decrease in AMP was observed. However, citrate levels rose nearly 3-fold in both hearts and livers of diabetic rats (91). Enzyme assays demonstrated that appropriate levels of citrate could indeed inhibit phosphofructokinase to the expected degree.

More recent studies have demonstrated that citrate has a differential inhibitory effect on the enzyme from different sources (54). Under his assay conditions (54), Kemp found citrate to be approximately six times as effective as an inhibitor of the muscle than of the liver enzyme. Citrate will inhibit muscle phosphofructokinase when ITP is used as phosphate donor instead of ATP (83). This effect has not been reported for the liver enzyme. However, at low levels of ATP (0.3 mM), citrate is a most effective inhibitor of the liver enzyme. Certainly, in the presence of physiological concentrations of ATP, citrate may be a potent inhibitor of the enzyme from both liver *and* muscle.

(iii) Phosphorylated glycolytic intermediates and P-creatine.

3-PGA, 2-PGA, 2,3-diPGA, PEP and P-creatine have all been reported to inhibit phosphofructokinase. Uyeda and Racker (49) reported that both P-creatine and PEP inhibited the skeletal muscle enzyme, although the reported $K_i$'s were in the millimolar range. Krzanowski and Matchinsky (59) examined the effect of all these compounds on phosphofructokinases from brain and muscle. In their assay system at pH 7.1, 3-PGA was an extremely potent inhibitor of both the brain and muscle enzymes, with a $K_i$ for both of approximately 0.01 mM. 2-PGA, 2,3-diPGA and PEP also were highly inhibitory, all having nearly the same $K_i$ for the brain enzyme, approximately 0.1 mM. P-creatine inhibited the brain enzyme more strongly than the muscle enzyme, the respective $K_i$'s being approximately 1.0 and 1.5 mM. The inhibitory action of all these compounds was greatly potentiated by increasing the ATP concentration, and was reduced by

increasing the F6P concentration. At pH 8.0, all the inhibitors, either singly or together, were ineffective.

Kemp's work (54) on the isoenzymes from liver and muscle is partly in disagreement with the above results. He found P-creatine to be ineffective as an inhibitor, while PEP inhibited only the muscle enzyme, and even this only slightly ($K_i$ > 2 mM). 3-PGA was a relatively stronger inhibitor of the muscle enzyme ($K_i$ < 1 mM) but not of the liver enzyme. In his study only 2,3-diPGA was a significant inhibitor of the enzyme. Kemp suggested that this may mean that the liver and erythrocyte enzymes are very similar, a suggestion later verified when he demonstrated that anti-sera to the liver enzyme removed 96% of the activity of erythrocyte phosphofructokinase (81).

(c) Modulation of Fructose-diphosphatase activity by effectors of phosphofructokinase.

Fructose-diphosphatase catalyzes the hydrolysis of FDP to F6P, i.e. the reversal of the phosphofructokinase reaction. It is found mainly in gluconeogenic tissues such as liver and kidney cortex, where its function is primarily to provide a means of bypassing the thermodynamically highly unfavourable reversal of the phosphofructokinase reaction. It is also found, however, in non-gluconeogenic tissues such as muscle. Although this thesis deals primarily with phosphofructokinase, it would clearly be too simplistic to ignore fructose-diphosphatase entirely. Below will be considered briefly some of the properties of muscle fructose-diphosphatase, as in this tissue the enzyme is considered to be present primarily to increase the sensitivity of regulation at the level of F6P phosphorylation (93).

Fructose-diphosphatase has a $K_m$ of only 2 μM for FDP. This ensures that the enzyme will always be active at physiological concentrations of substrate. AMP is inhibitory at physiological concentrations (94), whilst P-creatine and citrate are stimulatory (95). These effectors are

also regulators of phosphofructokinase, but change its activity in the opposite manner. $Ca^{2+}$ inhibits phosphofructokinase, but this occurs at non-physiological concentrations (58, 96, 97). It has recently been demonstrated, however, that $Ca^{2+}$ will also inhibit fructose-diphosphatase, at concentrations which are found in working muscle (97). The net effect of $Ca^{2+}$ therefore, will be to stimulate glycolysis during muscle work. The net effect of phosphofructokinase and fructose-diphosphatase having common but oppositely directed effector molecules is to ensure that, under any given metabolic state, the activity of one enzyme will predominate, without excessive hydrolysis of ATP.

Newsholme and Gevers (13) have emphasized the increased sensitivity conferred on weak effector signals by such a cycling system. From their examples, it is clear that small changes in concentration of a single effector molecule, which is common to both enzymes, but acts oppositely on each of them (i.e. AMP, P-creatine, citrate) will cause a much larger change in net flux than could occur in the absence of substrate cycling. Newsholme and Crabtree (93) have considered the role of fructose-diphosphatase in muscle, and concluded that its function is to provide cycling at the F6P-FDP locus, which will markedly increase the rate of glycolysis through this site when AMP levels rise during work. Clearly the effects of P-creatine and citrate could be explained in a similar manner.

(d) Mechanism of action of effector ligands

In this section, an attempt will be made to explain how the binding of effector molecules to phosphofructokinase relates to its observed activity patterns.

It is now clearly established that phosphofructokinase will readily associate and dissociate into various species which have molecular weights that are integral multiples of 180,000 daltons (11, 50, 65, 67, 68). At

neutral pH, the predominant species are those with molecular weights 180,000 and 360,000 daltons. At alkaline pH, the enzyme exists in a variety of aggregated forms, with molecular weights from 360,000 up to several million daltons having been observed. Evidence has accumulated which suggests that a major means of regulating phosphofructokinase activity *in vivo* may be by interconverting the active 360,000 dalton tetramer with the inactive 180,000 dalton dimer (65, 67). Early work on the stabilization of purified phosphofructokinase showed that many of the activators of the enzyme are stabilizers, whilst some inhibitors may cause inactivation (58, 98, 99). It is now thought that those molecules which stabilize phosphofructokinase in solution, do so by maintaining the equilibrium between the 360,000 dalton tetramer and the 180,000 dalton dimer in favour of the active form. Before considering in detail the means by which association/dissociation may be important as a regulatory mechanism, it is necessary to consider what is known about the binding of certain key effectors to the enzyme.

As discussed briefly before, Kemp and Krebs (69) examined the binding of various effectors to purified skeletal muscle phosphofructokinase. They found that one molecule each of F6P, AMP, cAMP and ADP bound per protomer of enzyme, but 3 molecules of ATP bound for the same amount of enzyme. AMP, cAMP, ADP and ATP apparently all bound competitively at the one site. Increasing the pH, or the presence of AMP or $PO_4^{2-}$, increased the affinity of the enzyme for F6P. Conversely, the presence of citrate decreased the affinity of the enzyme for F6P, but increased its affinity for ATP. On the basis of these results and their own, Paetkau *et al* (68) proposed that each protomer comprised 3 regulatory and 1 catalytic subunits. Mansour and coworkers (70, 71) reported slightly different binding data, which they felt were consistent with each protomer comprising 2 regulatory and 2 catalytic subunits. Although it is still not proven which model is

valid, the following facts are clear:

(i) each protomer of phosphofructokinase consists of catalytic and regulatory subunits; and

(ii) binding of the substrate F6P is promoted by positive effectors, whilst binding of ATP is promoted by the negative effector, citrate.

Lowry and Passoneau (58), on the basis of purely kinetic experiments, suggested the presence of between 7 and 12 binding sites for substrate and effector molecules. Their results are difficult to interpret quantitatively, however, as some of the data was collected from experiments conducted at pH 8.0. At this pH, binding patterns are altered considerably from those observed at neutral pH (70).

Mansour and Ahlfors (65), using the homogeneous sheep heart enzyme, have demonstrated that the interconversion of the 180,000 dalton dimer to the 360,000 tetramer is promoted by positive effectors. Incubation of the enzyme at acid pH caused dissociation into the inactive form, whereas incubation at alkaline pH in the presence of FDP and ATP caused reappearance of the active associated form. (Note — although ATP has been considered previously as an inhibitor, it is also a substrate and may be considered a positive effector at low concentrations.)

On the basis of results such as these, several authors have suggested a model for phosphofructokinase action, based upon an association/dissociation mechanism (65, 67, 100, 101). In essence the model states:

(i) the basic active unit of phosphofructokinase is the 360,000 dalton tetramer;

(ii) the active 360,000 dalton tetramer may be dissociated to an inactive 180,000 dalton dimer, either by lowering the pH, by dilution, or by incubation with negative effectors; and

(iii) conversion of the enzyme from the inactive dimer back to the basic active unit or to forms of even higher molecular weight, may be effected by raising the pH, increasing the protein concentration, or by incubation with certain positive effectors (see Figure 1-1).

The binding data of Kemp and Krebs (69), and Mansour *et al* (70, 71) can be explained in terms of this association/dissociation model, i.e.

(i) there are both substrate and allosteric binding sites on phosphofructokinase for MgATP (the absolute numbers have not been unequivocally determined);

(ii) there are only substrate binding sites for F6P;

(iii) binding of (Mg).ATP to its allosteric sites favours dissociation of the enzyme into the inactive form;

(iv) citrate binding increases the affinity for (Mg).ATP binding to its allosteric site, thus increasing the proportion of dissociated enzyme; and

(v) AMP, cAMP and ADP all compete with (Mg).ATP for binding at one of the allosteric sites and their binding blocks dissociation. Binding of these ligands to an allosteric site on the dissociated form of the enzyme causes reassociation, thus their apparent stimulatory effect.

Although unequivocal binding data is available only for the ligands considered above, it is reasonable to assume that other effector molecules could act in a similar fashion. This model then allows for the division of positive effectors into two groups: those which stabilize phosphofructokinase, and those which activate the enzyme without necessarily protecting it from inactivation. $NH_4^+$ appears to belong to

the latter group (58). Presumably it stimulates the enzyme by some entirely separate means, possibly by causing a conformational change in the active form of the enzyme.

At neutral or slightly lower pH, with ATP present at inhibitory concentrations, a sigmoidal curve is obtained when activity is plotted against increasing F6P concentration. Ahlfors and Mansour (102) have shown that photo-oxidation of the enzyme prevents ATP from binding at its allosteric sites. In addition, photo-oxidation destroys the sigmoidal response to increasing F6P concentration. This suggests that it is the binding of ATP to an allosteric site (or sites), which causes the sigmoidal response to F6P. As the sigmoidal response is due to cooperative interactions between F6P substrate sites, simultaneous binding of the two ligands at F6P substrate and ATP allosteric sites must be intimately involved. Indeed it has been clearly demonstrated that small increases in F6P concentration can be most effective in overcoming ATP inhibition (e.g. 56, 58). Additionally, when F6P is added to an ATP-inactivated enzyme, a slow increase in activity is observed (101). This would suggest that F6P is either directly or indirectly displacing ATP from its allosteric site(s). The role of association/dissociation phenomena in controlling enzyme activity has recently been reviewed by Mansour (103).

1.4.6 Alteration of *in vivo* concentrations of effector molecules, correlated with changes in phosphofructokinase activity

In this section, several brief examples will be given of experiments which have attempted to correlate changes in effector concentrations with altered metabolic flux and changes in phosphofructokinase activity.

Knowing that ACTH stimulates steroidogenesis, Bell *et al* (104) postulated that the increased energy demand for steroid hydroxylation upon administration of this hormone, was met by an increase in glycolysis, caused by direct activation of enzymes of the pathway. Using crossovers

of glycolytic intermediates in frozen rat adrenals as their definition of activation of individual enzymes, they reported a considerable increase in phosphofructokinase activity within two minutes of intravenous administration of ACTH. Concomitant with the increase in phosphofructokinase activity, the levels of ATP and citrate fell, whilst the levels of AMP and cAMP rose. Clearly the activation of phosphofructokinase can be explained by an effector-modulated deinhibition of the enzyme.

Hems and Brosnan (25) measured the levels of glycolytic intermediates and other metabolites in rat livers and kidneys, freeze-clamped at different time intervals during the onset of experimental ischaemia. In ischaemic kidneys, glycolytic flux increased four-fold (as determined by the increase in lactate) within 30 seconds of severing the blood supply. This was accompanied by a crossover which indicated stimulation of phosphofructokinase. Concomitantly, ATP levels fell, whilst those of AMP, $PO_4^{2-}$ and $NH_4^+$ rose. As discussed earlier (1.4.5(c)), AMP inhibits fructose-diphosphatase. Thus, in addition to activating phosphofructokinase, the increase in AMP levels will greatly reduce substrate cycling, thus further enhancing the stimulatory effect. It has not been possible to definitely prove that phosphofructokinase is regulatory in the ischaemic liver, as the massive liberation of glycogen which accompanies anoxia prevents a crossover being observed. However, in the experiment of Hems and Brosnan glycolytic flux increased, and changes in the effector molecules of phosphofructokinase similar to those found in kidney were observed. These changes would be sufficient to cause an activation of phosphofructokinase and an inhibition of fructose-diphosphatase, and can thus explain the increased flux rate to lactate.

The third example is deliberately chosen because it demonstrates, firstly, that activation and inhibition of phosphofructokinase can be

difficult to determine by examination of effectors alone and, secondly, that phosphofructokinase is not necessarily responsible for controlling glycolytic flux rates at all times.

Greenbaum *et al* (88) found that when rats were starved for 72 hours, hepatic glycolytic flux fell by a factor of 50. However, assay of glycolytic intermediates showed a crossover at phosphofructokinase which apparently indicated activation. Concomitantly, the levels of ATP and citrate fell, whilst the levels of AMP, cAMP, FDP and $NH_4^+$ rose. Although the changes in effectors were not large, they would certainly support the observation that phosphofructokinase was activated, but left unresolved the reason for apparent enzyme activation during glycolytic inhibition.

There is a possible way of reconciling this data. As stated earlier (Section 1.4.5(a)) F6P is most effective in overcoming citrate inhibition. After 72 hours of starvation, F6P had dropped by 60%, whilst the cytoplasmic citrate level remained high enough to become inhibitory in the presence of reduced F6P levels (88). Start and Newsholme (105) have suggested that this drop in F6P levels may be of great importance in the switching of metabolism from glycolysis to gluconeogenesis during starvation. Certainly Greenbaum *et al* (88) found a good correlation between the flux rate and the F6P content.

Phosphohexose-isomerase is a high activity enzyme, so the observed level of F6P will be dependent upon the rate at which G6P is formed from glucose. During starvation, circulating blood glucose concentrations fall, and the supply of glucose and the resulting low glucokinase activity become the rate-limiting factors.

## 1.5 LYMPHOID TISSUE GLYCOLYSIS

Of the major mammalian tissues, no systematic study has yet been made of the regulation of glycolysis in lymphocytes or in solid lymphoid

tissues. This omission is puzzling, as it has been reported that glycolysis plays a key role during the early stages of lymphocyte transformation (106). Although lymphocytes of various sizes are the *major* cell type found in the solid lymphoid organs (thymus, spleen and lymph nodes), macrophages and polymorphonuclear leucocytes are also present in significant numbers, particularly in the spleen, and it would not be surprising if different regulatory patterns were found to be associated with altered glycolytic rates in these three cell types. Nevertheless, in view of Suter's contention (20) that lymphocytes are the cell type principally responsible for the consumption and oxidation of glucose by rat spleen slices, the present work was undertaken to elucidate:

(i) whether phosphofructokinase plays a rate-limiting role in spleen similar to that demonstrated for other mammalian tissues; and

(ii) whether the allosteric properties of phosphofructokinase are so adapted to tissue function, that the lymphocyte response to immunological stimulation (increased glucose uptake) can be ascribed to its activation.

In the present work, parallel studies have been conducted on spleen and thymus, to enable a direct metabolic comparison to be made between the heterogeneous spleen, and the almost homogeneous thymus.

When small lymphocytes are stimulated by antigen, they undergo rapid transformation to blast cells that are capable of division and further differentiation. Most studies carried out to date on the activation of lymphocytes *in vitro* have used the plant mitogens phytohaemagglutinin (PHA) or concanavalin A (con A) as "antigen analogues". These agents cause non-specific stimulation of "resting" small lymphocytes, which then undergo a sequence of morphological changes (blastogenesis and

subsequent mitosis) that are indistinguishable from those caused in a much smaller proportion of the cell population by more highly specific antigens or by allogeneic cells. A detailed *biochemical* description of the activation process is not available at the present time, and its elucidation constitutes one of the central problems of cellular immunology. A sketchy outline of the early metabolic events associated with mitogen binding can be reconstructed from work reported in the literature, and is provided here to give a functional context to the experiments described below.

Among the earliest events which are reported to follow mitogen binding to lymphocytes, changes in cyclic nucleotide levels have been given most prominence, but the evidence available is contradictory: cyclic AMP levels have been reported not to change during the first five minutes (107), or to increase by up to 300% (108), whilst cyclic GMP levels are reported to increase several-fold (107). Membrane-associated events such as increased turnover of phosphotidyl inositol (109) and lecithin (110) have been correlated, in time, with permeability changes to molecules as diverse as $Ca^{2+}$ ions (111), hexoses (112), γ-amino butyric acid (113) and uridine (114). Within twenty minutes of PHA or Con A addition, glycolytic flux to lactate increases 2-3 fold (106) and the new flux rate may be sustained for up to 3 days in a simple physiological saline medium (184). In contrast to the accelerated glycolysis associated with release of respiratory inhibition (Pasteur effect), this effect occurs in the presence of undiminished or slightly stimulated respiration (116), and is reported to be sensitive to puromycin inhibition (117). Roos and Loos (106) have proposed that increased glycolysis is essential for lymphocyte transformation, as the transformation process is blocked when both glycolysis and respiration are inhibited, but still occurs if the inhibition is directed against respiration alone. Whether increased

glycolysis is required to provide energy for the transformation process, as is proposed, or whether it merely accompanies the increased glucose uptake needed to meet an essential requirement for pentose precursors but is not itself essential, is a point worthy of careful experimental consideration. Clearly such precursors are required within 24 hours of stimulation, as RNA and protein synthesis both reach a maximum at about this time (118), and DNA synthesis, whose onset is delayed for about 48 - 72 hours (118), would have a similar requirement.

The work described in this thesis is part of a continuing, collaborative project designed to elucidate the control mechanisms that are responsible for changing glycolytic flux in rat spleen and thymus. Culvenor (115) and Keig (119), in our laboratory, have used mitogenic stimulation of rat thymocytes as a model system to identify possible sites of control in the glycolytic pathway, whilst Suter (20) has made a detailed study of the oxidation of endogenous fuels by the rat spleen slice and has examined the way in which the pattern of oxidation is modified by glucose, lactate, ketone bodies and long-chain fatty acids added to the medium. In these experiments certain generalizations have been arrived at that apply with equal force to both tissues. (U-$^{14}$C)-glucose, at saturating concentrations (> 5 mM), can account, at most, for only 30 - 40% of the oxygen consumption of unstimulated lymphoid tissue, as assessed by the percentage dilution of the radioactivity appearing in the respired $CO_2$. Because this value has never been observed to exceed 45%, even after addition of Con A or phytohaemagglutinin at optimal concentrations, or when high concentrations of (U-$^{14}$C)-lactate or (U-$^{14}$C)-pyruvate are used as alternative fuels, Suter (20) has suggested that the low activity of pyruvate dehydrogenase limits the rate of oxidation of carbohydrate fuels. The reaction catalyzed by this enzyme complex appears to be grossly out of equilibrium in lymphoid tissues (20, 120).

Acetoacetate, which is more readily oxidized than glucose but which produces acetyl-CoA independently of pyruvate dehydrogenase, by-passes this rate-limiting step and can label up to 70% of the respiratory $CO_2$. Thus, in the absence of specific activation of pyruvate dehydrogenase, the early increase in glucose uptake associated with mitogen stimulation is likely to result in lactate formation rather than pyruvate oxidation and this is, in fact, the pattern of glucose utilization found when rat thymocytes and human blood lymphocytes are exposed to phytohaemagglutinin (115, 117).

In contrast, acetoacetate oxidation is unchanged when Con A is used to stimulate rat thymocytes (121), so the accelerating effect of mitogens on energy metabolism appears to be directed at a rate-limiting step in the *glycolytic* pathway that precedes pyruvate dehydrogenase, and that does not require a fall in respiration to bring about its activation. Results of these experiments will be presented in Chapter 4 of this thesis, where their direct bearing on the problem of glycolytic stimulation in lymphoid tissue and its relationship to the transformation of immuno competent cells will be discussed.

## 1.6 AIMS OF THIS PROJECT

In view of the possible importance of glycolysis as an energy source during the early stages of lymphocyte transformation, this project was initially planned with three main objectives:

(i) to systematically determine the regulatory sites of glycolysis in rat spleen and thymus, using the procedures developed by Newsholme and Gevers (13), in a variety of experimental situations designed to bring about marked changes in flux;

(ii) to undertake a partial purification and detailed kinetic study of phosphofructokinase from a major mammalian lymphoid organ (pig spleen) with a view to establishing whether there are tissue-specific differences in its response to substrates and allosteric modifiers that may help to explain the relationship between immunological stimulation and accelerated glycolysis; and

(iii) in light of this evidence, to consider whether, and to what extent, changes in the glycolytic flux in lymphoid tissues brought about by a variety of stimuli can be explained on the basis of the allosteric behaviour of phosphofructokinase.

Attention has been focused on phosphofructokinase in the present study because of its primary role as a glycolytic regulator in other mammalian tissues (see Section 1.3.5) and, under certain circumstances, in lymphoid tissues (Chapter 2), but this emphasis is not intended to prejudice the conclusions arrived at under objective (iii), and presented in the final chapter of this thesis.

# CHAPTER 2

# IDENTIFICATION OF THE REGULATORY SITES OF GLYCOLYSIS

# CHAPTER 2

# IDENTIFICATION OF THE REGULATORY SITES OF GLYCOLYSIS

## 2.1 INTRODUCTION

This chapter is devoted to determining the regulatory steps of glycolysis in rat spleen and thymus, in accordance with the principles discussed in Chapter 1. In addition to methods more commonly used for identifying regulatory loci (experimental ischaemia and the use of anaesthetics), we have utilized the immunological nature of spleen and thymus, and identified regulatory sites by activating lymphocytes either by addition of Concanavalin A (Con A) to lymphocyte suspensions, or by initiating the Graft-versus-Host reaction in rat spleen *in vivo*.

Concanavalin A is a globular protein extracted from the jack bean (*Canavalia ensiformis*), which has the ability to non-specifically stimulate "resting" small lymphocytes to undergo blastogenesis and subsequently, mitosis. One of the early events which occurs during transformation, is a stimulation of glycolysis (117). By measuring glycolytic and other intermediates during the first 4 hours of mitogen stimulation, it is possible to determine which loci mediate this activation process.

By contrast, the Graft-versus-Host reaction is a very *specific* immunological reaction, in which a graft with very similar but not identical genetic composition, reacts against a host, which is itself unable to react against the graft. We have conducted this reaction *in vivo*, by injecting splenic lymphocytes from a homozygous parent strain rat (AA) into the tail vein of an F1 hybrid (AxB). The "B" component of the host's genetic makeup is foreign to the graft lymphocytes and they are stimulated. The graft lymphocytes proliferate within the host's spleen, so by freeze-clamping this organ and measuring the levels of intermediates

within it at different time intervals after injection, it is possible to follow the course of this stimulation.

## 2.2 MATERIALS

### Reagents

D-Glucose was purchased from B.D.H. Co. Ltd., Poole, U.K. Sodium pyruvate (Type II) and cyclic AMP were purchased from the Sigma Chemical Co., St. Louis, Missouri. Butyl-PBD was from Koch-Light Laboratories, Colnbrook, U.K., whilst inulin-(carboxylic acid-$^{14}$C) was obtained from the Radiochemical Centre, Amersham, England. Adenine nucleotides, pyridine nucleotides and all substrates were purchased from Boehringer Mannheim Gmbh, West Germany. Glyceraldehyde-3-P was obtained as the diethylacetal derivative, and glyceraldehyde-3-P was released according to the manufacturer's instructions. The resulting solution was adjusted to pH 6.0 with $KHCO_3$, then frozen at -20°C. Under these conditions, the compound was stable for approximately one week. All other chemicals used were of the highest purity available.

### Enzymes

Hexokinase was obtained from either the Sigma Chemical Co. (Type III) or from Boehringer Mannheim. All other enzymes were from Boehringer Mannheim.

### Animals

Outbred male albino Wistar rats were used in all experiments unless indicated otherwise. They were obtained from the John Curtin School of Medical Research, A.N.U., at 3 weeks of age, and reared in this Department until used at 8 - 10 weeks of age. They were given food (Rural Rat and Mouse Cubes, Wagga Wagga, N.S.W.) and water *ad libitum* unless otherwise indicated. Lighting was maintained on a strict 12 hr. day:12 hr. night regime, and the termperature was thermostatically controlled at a constant 25°.

## 2.3 METHODS

### 2.3.1 Preparation of tissue extracts for enzyme assays

Spleen

Animals were killed by cervical dislocation and their spleens removed. These were finely minced with scissors, then dispersed in 3 volumes of a buffer containing 50 mM Triethanolamine-HCl, 3 mM EDTA and 100 mm KCl, and adjusted to pH 7.5 with 5N KOH. This suspension was homogenized with ten up-and-down strokes in an ice-cooled Teflon glass tissue homogenizer (type B - A.H. Thomas, Philadelphia, U.S.A.). The homogenate was centrifuged for 30 minutes at 40,000 x g (4°C) in a Sorvall RC2B centrifuge, and the resultant supernatant decanted and used for enzyme assay immediately. It was found during the course of this work, that glyceraldehyde-3-phosphate dehydrogenase was extremely labile, and showed no activity after the preparative procedure described above. To enable a realistic assessment of its activity to be made, it was decided to forgo the centrifugation described above, and instead, to centrifuge the homogenate for only 2 minutes in an Eppendorf Microfuge at 5,000 x g. It was thus possible to assay the activity of this enzyme within 5 minutes of killing the animal.

Thymus

Animals were killed as before and their thymi removed. These were minced coarsely, then homogenized with 2 volumes of buffer and centrifuged as described above. The pellet was dispersed in buffer, and samples were examined for surviving whole cells by light microscopy. It was estimated that by this method, 95% or more of the cells were disrupted. Glyceraldehyde-3-phosphate dehydrogenase was labile in this tissue also, and was prepared in the same way as the spleen enzyme.

2.3.2 Assay of enzyme activities

All assays were conducted on a Zeiss PMQII spectrophotometer, with a Beckman recorder attached. The basic assay buffer contained 50 mM Triethanolamine, 1 mM EDTA and 10 mM $MgCl_2$, adjusted to pH 7.2 with 5N KOH. This pH is lower than that used in comparable studies (e.g. 21, 22). It was chosen because,

(i) it is a more physiological pH, and should give a truer indication of possible *in vivo* activities, and

(ii) any regulatory properties that the individual enzymes may possess, will be seen more easily at this lower pH.

All enzymes were assayed by coupling product formation to a reaction which involves the oxidation or reduction of one of the pyridine nucleotides. This enables the enzyme activity to be measured by following the change in optical density recorded at wavelength 340 n.m. For all assays, reactions were initiated by adding enzyme extract to cuvettes of 1 cm light path, containing a final assay volume of 3.1 mls. For each enzyme, substrate concentrations were chosen to give maximal velocities. Besides the basic assay buffer, the following additions were made;

for Hexokinase determination:

0.5 mM glucose, 1.2 mM ATP, 3.0 U glucose-6-phosphate dehydrogenase, and 0.2 mM $NADP^+$;

Glucose-6-phosphate dehydrogenase:

0.8 mM G6P and 0.2 mM $NADP^+$;

Phosphohexose isomerase (back reaction only):

1.3 mM F6P, 3.0 U glucose-6-phosphate dehydrogenase and 0.2 mM $NADP^+$;

Phosphofructokinase:

3.1 mM F6P, 1.1 mM ATP, 2.5 U aldolase, 7.0 U triose-phosphate isomerase, 2.0 U α-glycerophosphate

dehydrogenase and 0.1 mM NADH;

Aldolase:

1.0 mM FDP, 7.0 U triose-phosphate isomerase, 2.0 U α-glycerophosphate dehydrogenase and 0.1 mM NADH;

Triose-phosphate isomerase:

0.8 mM glyceraldehyde-3-phosphate, 4.5 U α-glycerophosphate dehydrogenase and 0.1 mM NADH;

Glyceraldehyde-3-phosphate dehydrogenase:

0.5 mM glyceraldehyde-3-phosphate, 12 mM arsenate and 0.2 mM $NAD^+$;

Phosphoglycerate kinase (back reaction only):

3.1 mM 3-PGA, 1.6 mM ATP, 3.5 U glyceraldehyde-3-phosphate dehydrogenase and 0.1 mM NADH;

Phosphoglycerate mutase:

1.7 mM 3-PGA, 2.3 mM ADP, 0.5 mM 2,3-diPGA, 4.0 U enolase, 4.6 U pyruvate kinase, 4.0 U lactate dehydrogenase and 0.1 mM NADH;

Enolase:

1.7 mM 2-PGA, 2.3 mM ADP, 4.6 U pyruvate kinase, 4.0 U lactate dehydrogenase and 0.1 mM NADH;

Pyruvate kinase:

2.0 mM PEP, 2.3 mM ADP, 4.6 U lactate dehydrogenase and 0.1 mM NADH;

Lactate dehydrogenase:

3.0 mM pyruvate and 0.1 mM NADH.

Usually between 20 and 50 µL of enzyme solution was used. In most cases this gave optical density changes between 0.05 and 0.30/minute. For triose-phosphate isomerase, either 2 or 5 µL was added to initiate

the reaction. The enzymes were tested at at least 2 extract concentrations and in all cases except phosphofructokinase, the observed activity was proportional to the concentration of extract added. The deviation from linearity with phosphofructokinase was of the order of 25%. The higher activity is reported in this study. The problem of non-linearity is considered in more detail in Chapter 3.

The following points should be noted in relation to this study:

(i) all buffers used were pH-adjusted with KOH. This ensured that $K^+$ was saturating, which is supported by the observation that neither pyruvate kinase nor phosphofructokinase showed any activation if more $K^+$ was added; and

(ii) although 2,3-diPGA was added for the phosphoglycerate mutase reaction, it increased activity by only 10 - 20%. Clearly the tissue extract or substrates added contained enough of this cofactor to ensure maximal activity.

2.3.3 Freeze-clamping

In order to determine the *in vivo* concentrations of glycolytic and other intermediates, the technique of freeze-clamping was used (19). Animals were killed by cervical dislocation and either their spleens or thymi were rapidly removed. The organ was immediately crushed between aluminium tongs precooled to -180° in liquid nitrogen. On average, spleens were frozen within 5 - 10 seconds of the animal's death, thymi within 10 - 15 seconds. The frozen tissue was quickly weighed and immediately transferred to a porcelain mortar containing liquid nitrogen. Perchloric acid (3 ml of 12%) was added dropwise to the mortar and the frozen material ground to a powder and transferred quantitatively into an ice-cooled Teflon glass tissue homogenizer (A.H. Thomas, Philadelphia,

U.S.A.). The tissue was homogenized with ten up-and-down strokes as the material began to thaw. The homogenate was transferred quantitatively into 15 ml Corex centrifuge tubes and centrifuged at 45,000 x g for 15 minutes to remove the protein pellet. The pellets were dried for 16 hr. at 125° and weighed to determine the dry weight of perchloric acid-treated starting material.

The supernatants were decanted into graduated 10 ml stoppered tubes and adjusted to approximately pH 5.5 with $K_2CO_3$ (5M) using one drop of "Universal" indicator. The tubes were centrifuged at 27,000 x g for 10 minutes to remove the precipitated $KClO_4$. The supernatants were decanted and used immediately for assays of the unstable intermediates, or were stored at -180° under liquid nitrogen. The results are expressed on a fresh weight basis, using the dry weights of the individual samples, and a conversion factor determined by subjecting control spleens to an identical perchloric acid extraction step prior to drying.

### 2.3.4 Analytical methods

Concentrations of all intermediates and cofactors were determined enzymatically using a Zeiss PMQII spectrophotometer, or a Zeiss PMQII spectrofluorimeter with ZFM4 fluorescence attachment. Glucose (122), glycogen (123), lactate (124), ammonia (125), ATP (126), ADP and AMP (127) and inorganic phosphate (128) were determined spectrophotometrically using published methods. Pyruvate (129), the hexose-monophosphates (130), the triose-phosphates and FDP (131), and the phosphoglycerates and PEP (129) were determined fluorimetrically, using suitable adaptations of published methods (115).

### 2.3.5 Determination of the extracellular space

Clearly the entire mass of an organ is not composed solely of the intracellular space of the cells within it, — membranous tissues and the blood vessels supplying the organ will occupy a finite portion of that mass. There are two major reasons why an accurate assessment of the

extracellular space is important:

(i) it enables an accurate estimate to be made of the intracellular concentrations of the various intermediates, and

(ii) it enables an estimate to be made of the *intracellular* concentration of those metabolites which are found both intracellularly and in the circulating plasma, e.g. glucose and lactate.

Williams and Woodbury (132) have determined the extracellular space of rat liver, using *inulin*. Inulin is a polysaccharide molecule which penetrates cell membranes with great difficulty. When radioactive inulin is injected into an animal intravenously, and time is given for it to diffuse freely through the extracellular fluid, then by comparing the amount of label within the tissue with the concentration of label circulating in the blood plasma, an estimate can be made of the extracellular space within the tissue.

We have used the method of Williams and Woodbury (132), to estimate the extracellular spaces of spleen and thymus.

(a) Preparation of tissue samples

Bilateral nephrectomies of ether-anaesthetized animals were performed by Dr P. McCullagh of the John Curtin School of Medical Research, A.N.U. Four hours after the operation, experimental animals were injected with 0.5 ml of sterile 0.85% NaCl, containing 3μ Ci of $^{14}$C-inulin, via a cannula which had been inserted into the femoral vein at the time of operation. Animals were killed 30 - 60 minutes later, by stunning and subsequent severance of the carotid artery.

Blood was collected in heparinized tubes and centrifuged for 10 minutes at 16,000 x g. Plasma was separated from the cell pellets, and 0.03 ml volumes were digested for 15 hours in 1.0M piperidine at 60°.

Spleens, thymi and livers were removed from each animal and blotted gently before weighing. Samples were dried to constant weight to allow determination of total tissue water. Samples from spleen, thymi and livers (0.2 - 0.5 g) were digested in 1.0M piperidine for 30 hours at 60°.

(b) Determination of radioactivity

Samples of the spleen, thymus, liver and plasma digests were mixed with 10 mls of scintillation fluid (toluene:methoxyethanol:butyl-PBD; 600 ml:400 ml:6 g), placed in glass scintillation vials and counted in a Packard Tri-Carb Liquid Scintillation Counter (Model 3375) until total counts reached at least 10,000. Corrections were made for quenching, and the values were converted to disintegrations per minute, by comparison with a $^{14}$C-n-hexadecane standard.

(c) Calculation of the extracellular space

The inulin space was calculated from the following formula:

$$\text{Inulin space (\%)} = \frac{\text{d.p.m./g. wet wt. x 0.92}}{\text{d.p.m./ml. plasma}} \times 100;$$

where 0.92 is a correction for the difference in water concentration in plasma and extracellular fluid due to the higher protein level in the former. Hence the inulin space represents the percentage of tissue weight in which inulin was distributed at a concentration similar to that found in the plasma.

(d) Metabolite concentrations in rat plasma

These determinations were made by Mrs G. Keig. Blood was collected into heparinized tubes and plasma separated from cells by centrifugation at 800 x g in the cold. Very little or no haemolysis was evident in any of the samples. Plasma samples were prepared for assay as described in Section 2.3.3. The only metabolites of which significant quantities were found in plasma, were glucose, pyruvate, lactate, phosphate and ammonia.

The values quoted in the Results section have all been corrected for these intermediates.

(e) Calculations of intracellular metabolite concentrations

Intracellular concentrations of glycolytic intermediates, adenine nucleotides, inorganic phosphate and ammonia in rat spleen and thymus were calculated in the following manner:

(i) The extracellular concentration of a particular metabolite was assumed to be equal to its concentration in plasma. Then,

(ii) Extracellular metabolite (n. moles/g. wet wt.) = extracellular metabolite concentration (n. moles/ml extracellular water) x volume of extracellular water (ml/g. wet wt.).

(iii) Intracellular metabolite (n. moles/g. wet wt.) = total tissue metabolite (n. moles/g. wet wt.) - extracellular metabolite (n. moles/g. wet wt.).

(iv) Intracellular metabolite concentration (n. moles/ml intracellular water) = intracellular metabolite (n. moles/g. wet wt.) ÷ volume of intracellular water (ml/g. wet wt.).

The total metabolite concentrations in spleen and thymus were obtained from freeze-clamping experiments, as described in Sections 2.3.3 and 2.3.4.

2.3.6 Preparation and incubation of thymocytes for measurement of Concanavalin A-stimulation

Thymi were removed from 7-week old male albino Wistar rats, weighed, then teased apart with 22-gauge needles in Petri dishes containing a small amount of Krebs and de Gasquet buffer, pH 7.4 (133). Large debris was removed by spinning in a bench centrifuge for 30 seconds at 50 x g, then the supernatants were centrifuged for 7 minutes at 800 x g to pellet the

thymocytes. These were resuspended in buffer with a Pasteur pipette, pooled, and after counting in a haemocytometer, were diluted with buffer to the appropriate concentrations.

Cells were incubated in 25 ml Erlenmeyer flasks with rubber stoppers, at final concentrations of $10^8$ cells/ml in 5 mls of buffer, in the presence of 5 mM glucose. After gassing with oxygen for 30 seconds, cells were incubated at 37° in a shaking water bath. After a one hour preincubation at 37° to allow complete equilibration, experiments were commenced. Zero time samples were taken at the time of addition of Concanavalin A. The final concentration of mitogen in Concanavalin A-treated suspensions, was 20 µg/$10^7$ cells.

At the end of the incubation period, 0.5 ml of ice-cold 3N perchloric acid was added. After standing on ice for at least 30 minutes, the contents of the flasks were transferred to 15 ml Corex centrifuge tubes and centrifuged for 15 minutes at 35,000 x g in a Sorvall RC2B centrifuge. The supernatant was treated as described in Section 2.3.3 for preparing the intermediates. Subsequent assays were performed as in Section 2.3.4.

## 2.4 RESULTS

### 2.4.1 Determination of the extracellular spaces

All the calculations reported in this section were made by Mrs G. Keig. Although interested primarily in the results for spleen and thymus, the liver space was also determined to permit comparison with published results. The one-hour inulin space for rat liver was calculated to be 18.4%. By contrast, Williams and Woodbury (132) reported a space of only 10%, whilst White and Rolf (134) reported a space of 14.3%.

The higher value of 18.4% obtained in the present study can be attributed to the different methods used for drawing blood and killing the animals. White and Rolf (134) withdrew blood from the heart (presumably

under ether anaesthesia) and described the organs removed for analysis as "almost bloodless". Williams and Woodbury (132) withdrew blood from the terminal aorta after ether anaesthesia, and reported a low residual blood volume. However, ether anaesthesia has been found to alter the distribution of water between the plasma and extracellular compartments (135), and this introduces a source of error into isotope dilution studies (136).

The one hour inulin space for rat spleen was calculated to be 17.2%. There was more variation in the spleen inulin spaces of the six animals than in the liver inulin spaces. This must be attributed to variations in the amount of residual blood in the individual spleen samples. The connective tissue framework of the spleen collapses upon death, compressing the red pulp, and forcing blood out of the venous sinusoids (137). For this reason, a more marked variation between animals for spleen inulin spaces would be expected as a result of differences in the times taken for removal of the spleen and preparation of samples. These differences would lead to varying amounts of blood loss from the red pulp.

The one hour inulin space for rat thymus was calculated to be 12.4%. As the intravescular space of the thymus is only about one-fifth that of the spleen (138), it is to be expected that its inulin space would also be smaller.

### 2.4.2 Maximal enzyme activities *in vitro*

In Tables 2.1 and 2.2, the maximal activities of glycolytic and related enzymes in rat spleen and thymus are shown. It is evident that in both tissues hexokinase, phosphofructokinase, aldolase and glucose-6-phosphate dehydrogenase have much lower activities than the other enzymes. Suter, however (20), has shown that the maximum activity of each of these low activity enzymes is approximately 10 times greater than the maximal rate of glucose utilization observed in incubated spleen slices. Clearly

Table 2.1 Maximal activities of glycolytic and related enzymes in rat spleen

Enzyme activities are expressed as μ moles substrate converted/min/g fresh wt at 23°, ± S.E.M.
The number of determinations are given in parentheses.

| ENZYME | Km (μM) | | ACTIVITY (fed) | | ACTIVITY (fasted 72 hr) | |
|---|---|---|---|---|---|---|
| Hexokinase | <20 | (glucose) | 0.8±0.1 | (6) | 0.8±0.1 | (5) |
| Phosphohexose-Isomerase (back) | 105 | (back) | 31.5±1.3 | (5) | 26.9±1.0 | (6) |
| Phosphofructokinase | 45<br>130 | (ATP)<br>(F6P) | 1.6±0.1 | (8) | 1.3±0.1 | (6) |
| Aldolase | 15 | | 1.6±0.2 | (6) | 1.4±0.1 | (5) |
| Triose-phosphate Isomerase | — | | 183 ±6 | (3) | — | |
| Glyceraldehyde-3-phosphate dehydrogenase | — | | 15.8±0.3 | (3) | — | |
| Phosphoglycerate kinase (back) | 110 | (back) | 64.9±2.8 | (5) | 64.0±2.1 | (6) |
| Phosphoglycerate mutase | 215 | | 39.3±1.9 | (5) | 39.7±1.5 | (6) |
| Enolase | 100 | | 13.1±0.8 | (6) | 14.1±0.8 | (6) |
| Pyruvate kinase | 30 | (PEP) | 45.8±0.8 | (7) | 47.4±1.2 | (5) |
| Lactate dehydrogenase | 125 | | 89.5±2.7 | (7) | 84.2±2.4 | (7) |
| Glucose-6-phosphate dehydrogenase | 40 | | 6.8±0.6 | (6) | 6.3±0.2 | (5) |
| Fructose-diphosphatase | — | | 0.2 | (3) | — | |

Table 2-2

Maximal activities of glycolytic and related enzymes in rat thymus

Enzyme activities are expressed as in Table 2-1.

| ENZYME | ACTIVITY (Fed) | |
|---|---|---|
| Hexokinase | 1.0 ± 0.1 | (4) |
| Phospho-hexose Isomerase (back) | 30.8 ± 4.0 | (4) |
| Phosphofructokinase | 2.2 ± 0.1 | (4) |
| Aldolase | 1.1 ± 0.1 | (4) |
| Triose-phosphate isomerase | 104.2 ± 8.2 | (3) |
| Glyceraldehyde-3-phosphate dehydrogenase | 21.0 ± 0.5 | (3) |
| Phosphoglycerate kinase (back) | 49.9 ± 3.0 | (4) |
| Phosphoglycerate mutase | 38.5 ± 1.3 | (4) |
| Enolase | 18.7 ± 2.2 | (4) |
| Pyruvate Kinase | 38.7 ± 2.3 | (4) |
| Lactate dehydrogenase | 73.8 ± 9.0 | (4) |
| Glucose-6-phosphate dehydrogenase | 3.1 ± 0.1 | (4) |
| Fructose-diphosphatase | 0 | (4) |

no enzyme of this series is rate-limiting with respect to its maximum activity. In addition to their low maximal activities, hexokinase, phosphofructokinase and glucose-6-phosphate dehydrogenase all catalyze reactions at sites that are teleologically advantageous for regulation. In Figure 2-1, the data in Tables 2-1 and 2-2 is expressed in diagramatical form. This layout emphasizes the low activities of both hexokinase and phosphofructokinase, and the potential they may possess for causing "bottle-necks" in flux.

It is interesting that a very low fructose-diphosphatase activity can be measured in spleen. Shonk and Boxer (22) also found a measurable activity for this enzyme. Suter, however (20), observed that after incubation of spleen slices with radioactive pyruvate or lactate, only very low levels of labelled glucose were recovered. It may well be that fructose-diphosphatase exists in spleen as it does in muscle (93), to provide substrate cycling between F6P and FDP, thus enhancing flux through phosphofructokinase when AMP levels rise.

Table 2-1 also presents data showing the effect of 72 hours of starvation on the maximal activities of the glycolytic enzymes. Although several of the changes approach significance at the 5% level, it is evident that spleen and thymus metabolism too, is rather insensitive to dietary manipulation. Even fasting and refeeding (data not shown) caused little change.

### 2.4.3 Identification of non-equilibrium reactions

In Tables 2-3 and 2-4 the *in vivo, intracellular* concentrations of intermediates in rat spleen and thymus are listed. Those intermediates of which significant quantities were found in plasma (see Section 2.3.5(d)) have been corrected to allow determination of their intracellular concentrations.

Figure 2-1 Maximal activities of glycolytic enzymes

(a) Spleen

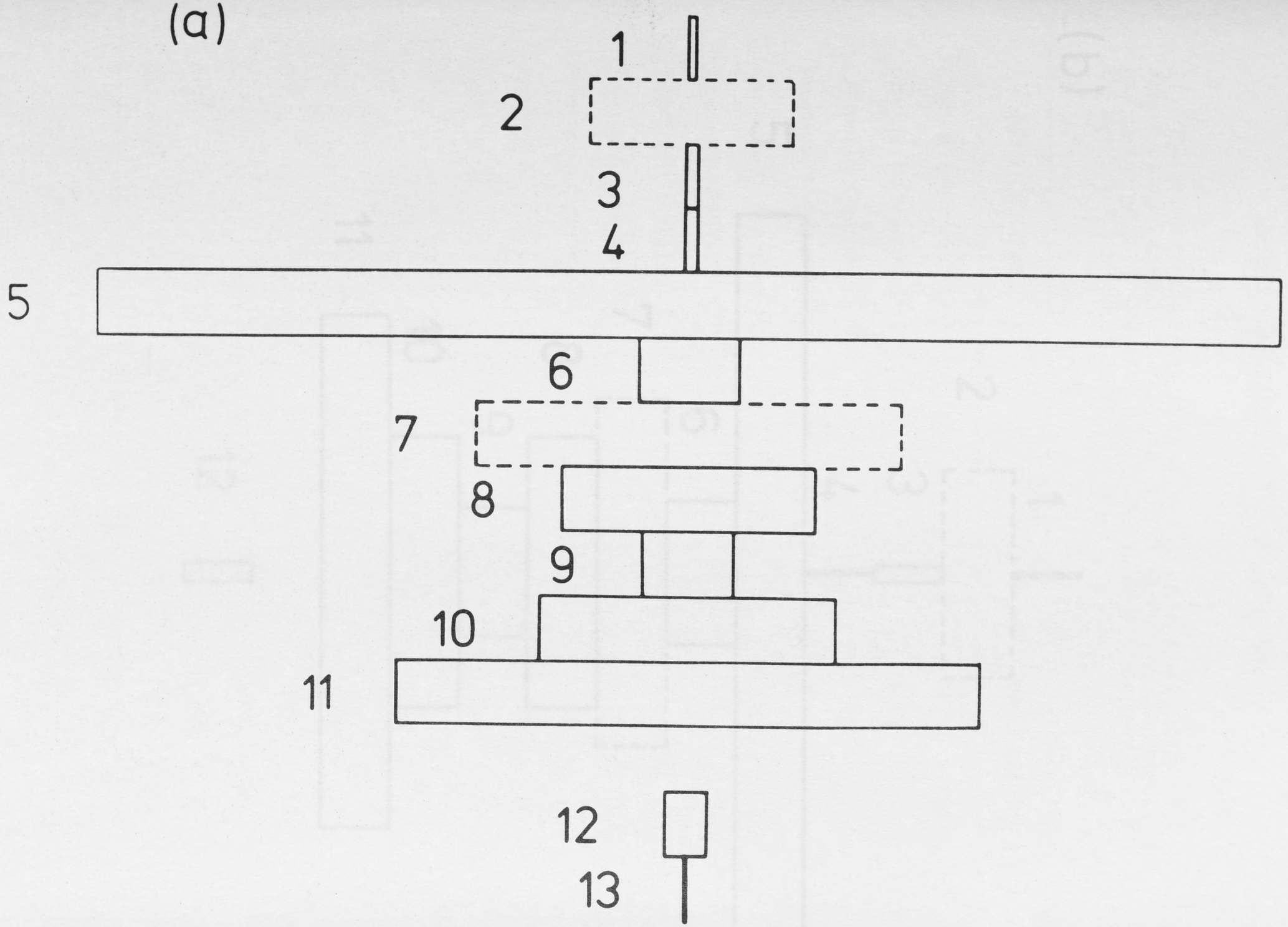
(b) Thymus

Enzyme activities were drawn from Tables 2-1 and 2-2. Activities represent the forward reactions unless indicated by *dashed* lines. For reference, the activity of spleen hexokinase is 0.8, and thymus hexokinase 1.0 μ moles substrate converted/min./g. fresh wt., at pH 7.2.

KEY

1. Hexokinase
2. Phosphohexose isomerase
3. Phosphofructokinase
4. Aldolase
5. Triose phosphate isomerase
6. Glyceraldehyde-3-phosphate dehydrogenase
7. Phosphoglycerate kinase
8. Phosphoglycerate mutase
9. Enolase
10. Pyruvate kinase
11. Lactate dehydrogenase
12. Glucose-6-phosphate dehydrogenase
13. Fructose-diphosphatase

(a)



(b)

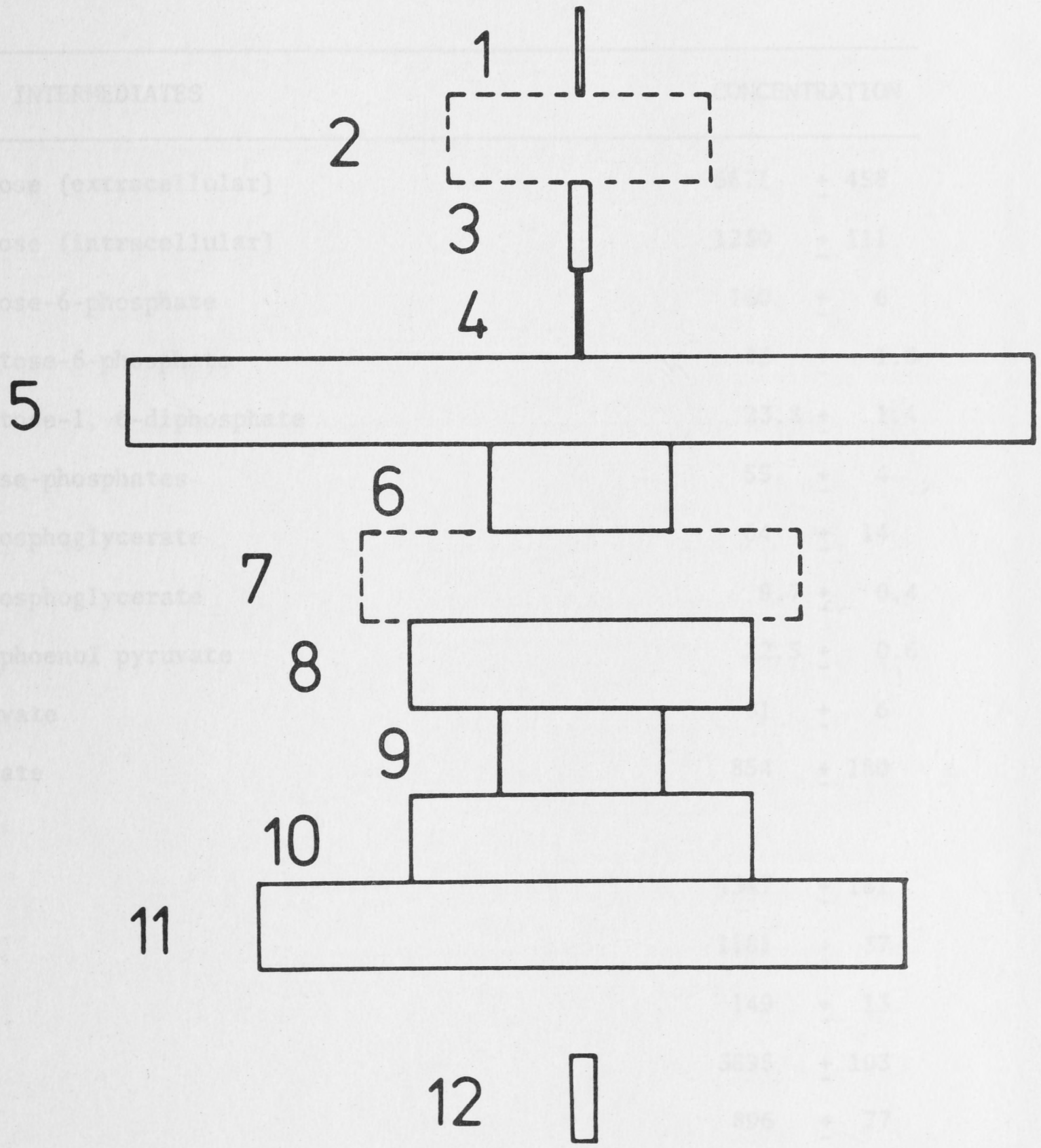


Table 2-3

The *in vivo* concentrations of glycolytic and other intermediates found in the spleen of the well-fed rat

The intermediates were assayed in extracts prepared from the freeze-clamped tissues as described in the text. The concentrations are expressed as n. moles/ml intracellular water. All results are the means ± S.E.M. of at least 10 values.

| INTERMEDIATES | CONCENTRATION |
|---|---|
| Glucose (extracellular) | 6671 ± 458 |
| Glucose (intracellular) | 1230 ± 111 |
| Glucose-6-phosphate | 160 ± 6 |
| Fructose-6-phosphate | 45 ± 1.8 |
| Fructose-1, 6-diphosphate | 23.8 ± 1.4 |
| Triose-phosphates | 55 ± 4 |
| 3-phosphoglycerate | 64 ± 14 |
| 2-phosphoglycerate | 9.7 ± 0.4 |
| Phosphoenol pyruvate | 22.5 ± 0.6 |
| Pyruvate | 81 ± 6 |
| Lactate | 854 ± 180 |
| ATP | 4347 ± 181 |
| ADP | 1181 ± 37 |
| AMP | 149 ± 13 |
| Pi | 3895 ± 103 |
| $NH_4^+$ | 896 ± 77 |
| Citrate | 213 ± 24 |

Table 2-4

The *in vivo* concentrations of glycolytic and other intermediates found in the thymus of the well-fed rat

The intermediates were assayed in extracts prepared from the freeze-clamped tissues as described in the text. The concentrations are expressed as n. moles/ml intracellular water. All results are the means ± S.E.M. of at least 8 values.

| INTERMEDIATES | CONCENTRATION |
|---|---|
| Glucose (extracellular) | 6710 ± 458 |
| Glucose (intracellular) | 720 ± 204 |
| Glucose-6-phosphate | 33.1 ± 6.6 |
| Fructose-6-phosphate | 10.9 ± 1.1 |
| Fructose-1, 6-diphosphate | 41.0 ± 7.4 |
| Triose-phosphates | 37.6 ± 7.1 |
| 3-phosphoglycerate | 38.9 ± 7.5 |
| 2-phosphoglycerate | 7.8 ± 2.1 |
| Phosphoenol pyruvate | 11.5 ± 2.5 |
| Pyruvate | 89.8 ± 31 |
| Lactate | 2155 ± 255 |
| ATP | 3980 ± 184 |
| ADP | 1482 ± 85 |
| AMP | 289 ± 20 |
| Pi | 3261 ± 182 |
| $NH_4^+$ | 1326 ± 113 |
| Citrate | 245 ± 10 |

In Table 2-5 the mass action ratios for the glycolytic enzymes in spleen and thymus, and their apparent equilibrium positions are presented. For ease of identification of enzymes catalyzing non-equilibrium reactions, the ratio $\frac{\Gamma}{K'}$ has been used. The significance of this ratio is as follows:

$$\frac{\Gamma}{K'} = 1, \quad \text{reaction at equilibrium.}$$

$$\frac{\Gamma}{K'} > 1, \quad \text{reaction in favour of products.}$$

$$\frac{\Gamma}{K'} < 1, \quad \text{reaction in favour of reactants.}$$

It is clear that in both tissues phosphohexose-isomerase, phosphoglycerate mutase and enolase catalyze equilibrium reactions in the unstimulated, well-fed rat, whilst the hexokinase, phosphofructokinase and pyruvate kinase reactions are significantly displaced from equilibrium. The reaction catalyzed by aldolase is also out of equilibrium, but to a lesser degree. Estimation of the intracellular and extracellular concentrations of glucose, show that the glucose carrier is in the intermediate range ($0.05 < \frac{\Gamma}{K'} < 0.20$) that Rolleston (15) considers separates non-equilibrium from equilibrium reactions.

Several of the mass-action ratios were calculated by indirect means. It is not possible to measure quantities of 1,3-diPGA in these tissues, so it is not possible to determine separate mass-action ratios for glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. Instead, a combined mass-action ratio for these two reactions has been calculated. However, even this value is based upon an assumption as only *total* triose-phosphates were measured, without differentiating between dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Triose-phosphate

Table 2.5 Comparison between mass-action ratios and equilibrium constants for the reactions of glycolysis in spleen and thymus

Mass-action ratios were calculated from the concentrations of intermediates given in Table 2.4.
Intracellular phosphate was assumed equal to 60% of the measured value (153).
Triose-phosphate isomerase was assumed to be at equilibrium with a value of 0.0408 for its apparent equilibrium constant.
Apparent equilibrium constants were taken from the literature quoted, and a mean determined for those considered most reliable.

| Enzyme Reaction | Ratio used | Apparent equilibrium constant (K') | Source | Spleen mass-action ratios observed (Γ) | Spleen $\frac{\Gamma}{K'}$ | Thymus mass-action ratios observed (Γ) | Thymus $\frac{\Gamma}{K'}$ |
|---|---|---|---|---|---|---|---|
| Glucose transport | $\frac{[Glucose]_{in}}{[Glucose]_{out}}$ | 1.0 | 151 | 0.184 | 0.184 | 0.107 | 0.107 |
| Hexokinase | $\frac{[G6P][ADP]}{[Glucose]_{in}[ATP]}$ | 4700 | 28,152 | $3.53 \times 10^{-2}$ | $7.5 \times 10^{-6}$ | $1.7 \times 10^{-2}$ | $3.64 \times 10^{-6}$ |
| Phosphohexose isomerase | $\frac{[F6P]}{[G6P]}$ | 0.41 | 28,152 | 0.281 | 0.686 | 0.329 | 0.803 |
| Phosphofructokinase | $\frac{[FDP][ADP]}{[F6P][ATP]}$ | 1003 | 28,152,154 | 0.144 | $1.43 \times 10^{-4}$ | 1.40 | $1.4 \times 10^{-3}$ |
| Aldolase | $\frac{[G3P][DHAP]}{[FDP]}$ | $9.6 \times 10^{-5}$ | 28,152,155 | $4.8 \times 10^{-6}$ | $5.0 \times 10^{-2}$ | $1.35 \times 10^{-4}$ | $1.4 \times 10^{-2}$ |
| Glyceraldehyde-3-phosphate dehydrogenase + phospho-glycerate kinase | $\frac{[NADH][3PGA][ATP]}{[NAD^+][G3P][ADP][Pi]}$ | 59 | 156 | $1.09 \times 10^{2}$ | 1.85 | 55.6 | 0.942 |
| Phosphoglycerate mutase | $\frac{[2PGA]}{[3PGA]}$ | 0.149 | 28,152,155 | 0.151 | 1.01 | 0.201 | 1.35 |
| Enolase | $\frac{[PEP]}{[2PGA]}$ | 2.93 | 28,152,155 | 2.32 | 0.79 | 1.47 | 0.503 |
| Pyruvate kinase | $\frac{[Pyr][ATP]}{[PEP][ADP]}$ | 17,900 | 28,152 | 13.3 | $7.4 \times 10^{-4}$ | 21.0 | $1.17 \times 10^{-3}$ |
| Lactate dehydrogenase | $\frac{[Lactate][NAD^+]}{[Pyruvate][NADH]}$ | 11,100 | 157 | | assumed at equilibrium | | |
| Adenylate kinase | $\frac{[ATP][AMP]}{[ADP]^2}$ | 0.44 | 158 | 0.46 | 1.06 | 0.524 | 1.19 |

isomerase is the most active enzyme in both of these tissues, and if it is assumed to be at equilibrium (154), the concentrations of the individual triose-phosphates can be calculated. This in turn enables the calculation of mass-action ratios for the combined glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase reactions, and for aldolase. The value of the free $NAD^+$/NADH ratio for the cytoplasmic compartment was calculated from the lactate/pyruvate ratio, on the assumption that the lactate dehydrogenase reaction is sufficiently active to maintain equilibrium.

Figure 2-2 shows the free energy changes for the individual reactions of glycolysis in both spleen and thymus. Values of $\Delta G$ were calculated from the mass-action ratios and apparent equilibrium constants in Table 2-5, by the following relationship derived from Morris (139).

$$\Delta G = \Delta G^\circ + R.T. \ln \Gamma.$$

and since $\Delta G^\circ = - R.T. \ln K'$,

$$\Delta G = - R.T. \ln \left(\frac{K'}{\Gamma}\right) \text{ K. cals./mole,}$$

where $\Delta G$ = free energy change at 37°, and pH assumed to be 7.0,

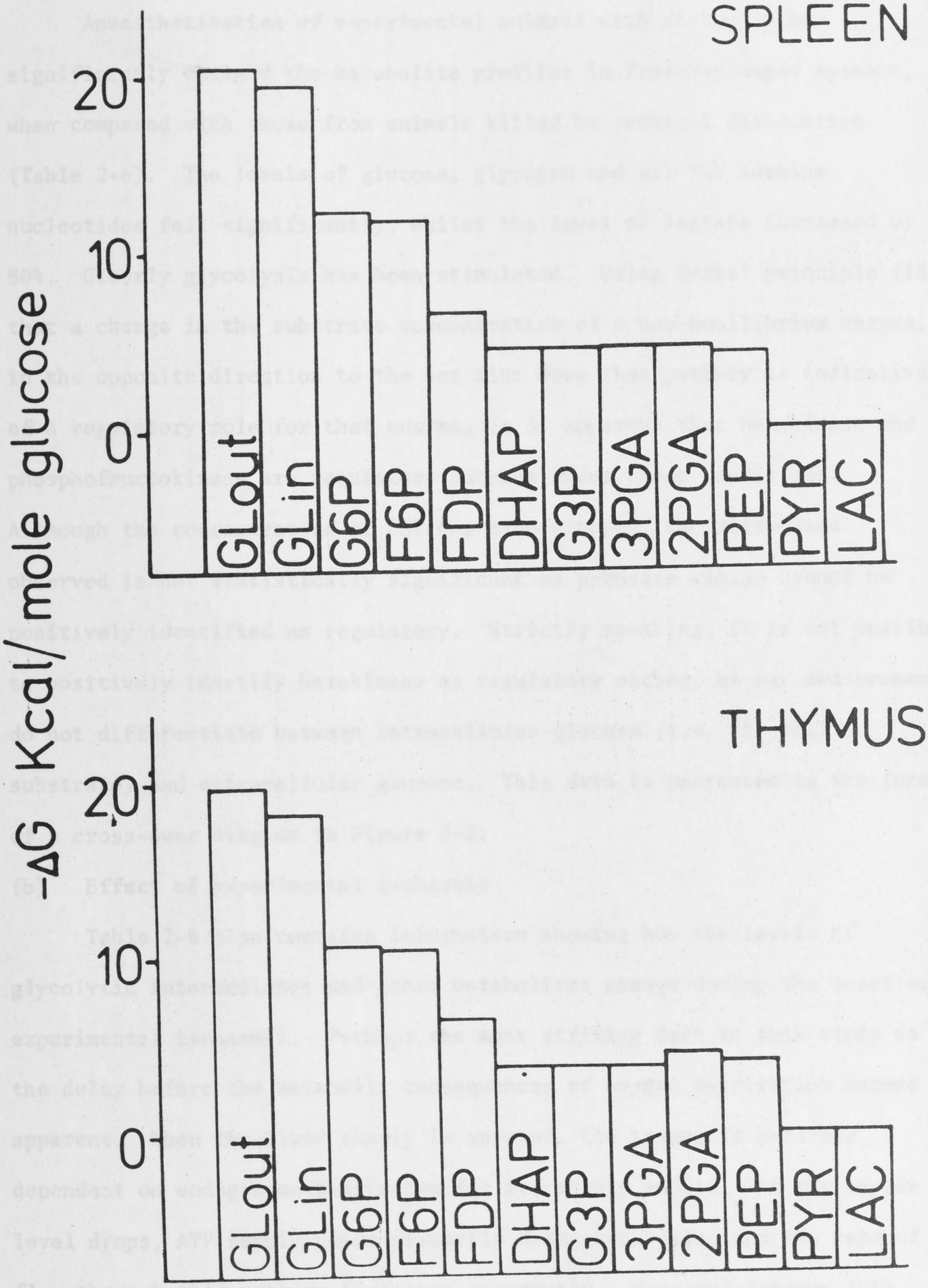
$R$ = universal gas constant (1.987 cal./mole degree),

and $T$ = temperature, degrees absolute.

Most of the free energy change during glycolysis occurs in both tissues at the reactions catalyzed by hexokinase, phosphofructokinase and pyruvate kinase. In addition, significant changes in free energy occur during glucose transport and at the reaction catalyzed by aldolase.

Figure 2-2 Relative free energy changes during the conversion of glucose to lactate, in spleen and thymus

ΔG values were calculated as indicated in the text.


SPLEEN
THYMUS
-ΔG Kcal/ mole glucose
20
10
0
GLout
GLin
G6P
F6P
FDP
DHAP
G3P
3PGA
2PGA
PEP
PYR
LAC

2.4.4 Identification of regulatory sites

(a) Effect of ether anaesthesia

Anaesthetisation of experimental animals with diethyl ether significantly changed the metabolite profiles in freeze-clamped spleens, when compared with those from animals killed by cervical dislocation (Table 2-6). The levels of glucose, glycogen and all the adenine nucleotides fell significantly, whilst the level of lactate increased by 80%. Clearly glycolysis has been stimulated. Using Krebs' principle (18) that a change in the substrate concentration of a non-equilibrium enzyme, in the opposite direction to the net flux down that pathway is indicative of a regulatory role for that enzyme, it is apparent that hexokinase and phosphofructokinase are regulatory enzymes under these conditions. Although the concentration of PEP has also dropped, the difference observed is not statistically significant so pyruvate kinase cannot be positively identified as regulatory. Strictly speaking, it is not possible to positively identify hexokinase as regulatory either, as our measurements do not differentiate between intracellular glucose (i.e. the real substrate) and extracellular glucose. This data is presented in the form of a cross-over diagram in Figure 2-3.

(b) Effect of experimental ischaemia

Table 2-6 also contains information showing how the levels of glycolytic intermediates and other metabolites change during the onset of experimental ischaemia. Perhaps the most striking fact in this study is the delay before the metabolic consequences of oxygen deprivation become apparent. When the blood supply is severed, the tissue is entirely dependent on endogenous substrates for its energy supply. As the oxygen level drops, ATP supply comes primarily from glycolysis, and the rate of flux through this pathway increases enormously. Hems and Brosnan (25) found the onset of ischaemia in liver and kidney to be comparatively rapid,

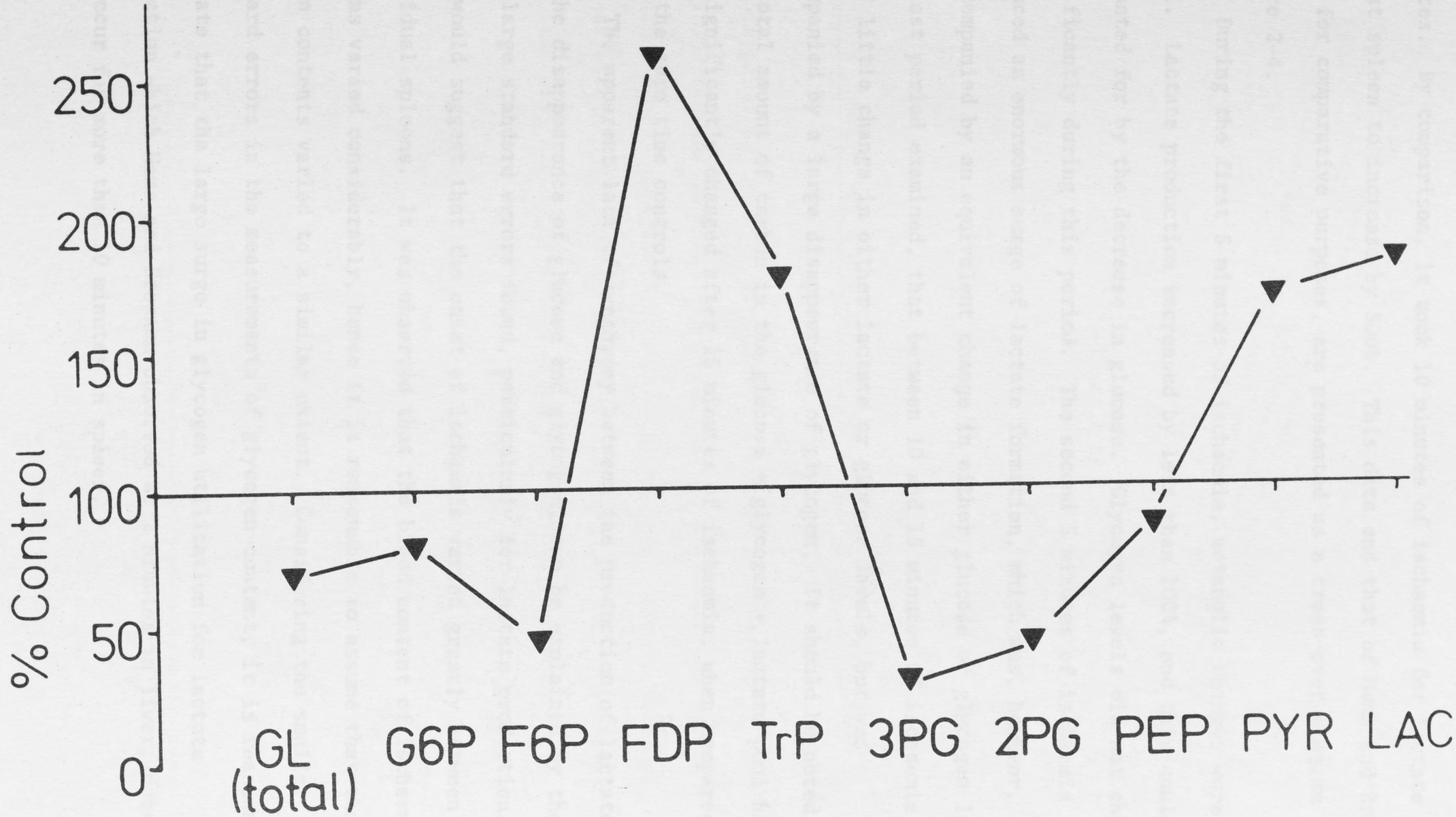
Table 2.6 Concentrations of glycolytic and other intermediates in the spleen of well-fed rats during ether anaesthesia and during the onset of ischaemia

The intermediates were assayed in extracts prepared from the freeze-clamped tissue as described in the text.
The concentrations are expressed as n moles/g fresh wt.
All results are the means ± S.E.M. of at least 5 values.

| Time after cutting vessels (min.) | Treatment of animal | Glyc. | Gluc. (Total) | G-6-P | F-6-P | FDP | TP | 3PGA | 2PGA | PEP | Pyr. | Lac. | αGP | ATP | ADP | AMP | Total Adn. | Energy charge | $NH_4^+$ | Pi |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | Ether anaesthesia | 2334 ±504 | 1469 ±180 | 78 ±15 | 12 ±6 | 36 ±14 | 58 ±23 | 11 ±13 | 3 ±4 | 12 ±7 | 136 ±49 | 1545 ± 170 | 51 ±18 | 1722 ±219 | 428 ±121 | 45 ±25 | 2189 ±352 | 0.89 ±0.02 | 654 ± 284 | — |
| 0 | Cervical dislocation | 3390 ±280 | 2108 ±180 | 97 ± 4 | 27 ±0.9 | 14 ± 0.7 | 33 ± 2 | 39 ± 7 | 7 ±0.4 | 14 ±0.4 | 81 ± 6 | 854 ± 180 | 96 ± 4 | 2619 ± 94 | 712 ± 30 | 91 ±10 | 3422 ±128 | 0.87 ±0.003 | 567 ± 50 | 2581 ± 77 |
| 5 | Cervical dislocation | 2930 ±210 | 1686 ± 46 | 75 ± 3 | 22 ±0.3 | 9 ± 1 | 28 ± 2 | 31 ± 3 | 8 ±1 | 9 ±1.2 | 68 ± 7 | 1612 ± 31 | 114 ± 7 | 2148 ± 60 | 686 ± 26 | 167 ±11 | 3001 ± 80 | 0.83 ±0.006 | 1045 ± 231 | 2725 ±130 |
| 10 | Cervical dislocation | 3070 ±290 | 1183 ±204 | 19 ± 1.2 | 6 ±0.5 | 32 ± 5 | 68 ±10 | 33 ± 4 | 11 ±0.6 | 12 ±1 | 61 ± 2 | 4590 ±1288 | 349 ±60 | 1169 ±111 | 856 ± 45 | 579 ±98 | 2605 ±104 | 0.62 ±0.038 | 2050 ±1234 | 4911 ±649 |
| 15 | Cervical dislocation | 1840 ±410 | 900 ±110 | | | | | | | | | 4887 ±1047 | | | | | | | | |

Figure 2-3 "Crossover" plot of the anaesthetised rat spleen

The levels of glycolytic intermediates in the anaesthetised rat spleen are plotted as a percentage of the control *in vivo* values. The levels of the intermediates in the control are expressed as 100%.


250
200
150
100
50
0
% Control
GL (total)
G6P
F6P
FDP
TrP
3PG
2PG
PEP
PYR
LAC

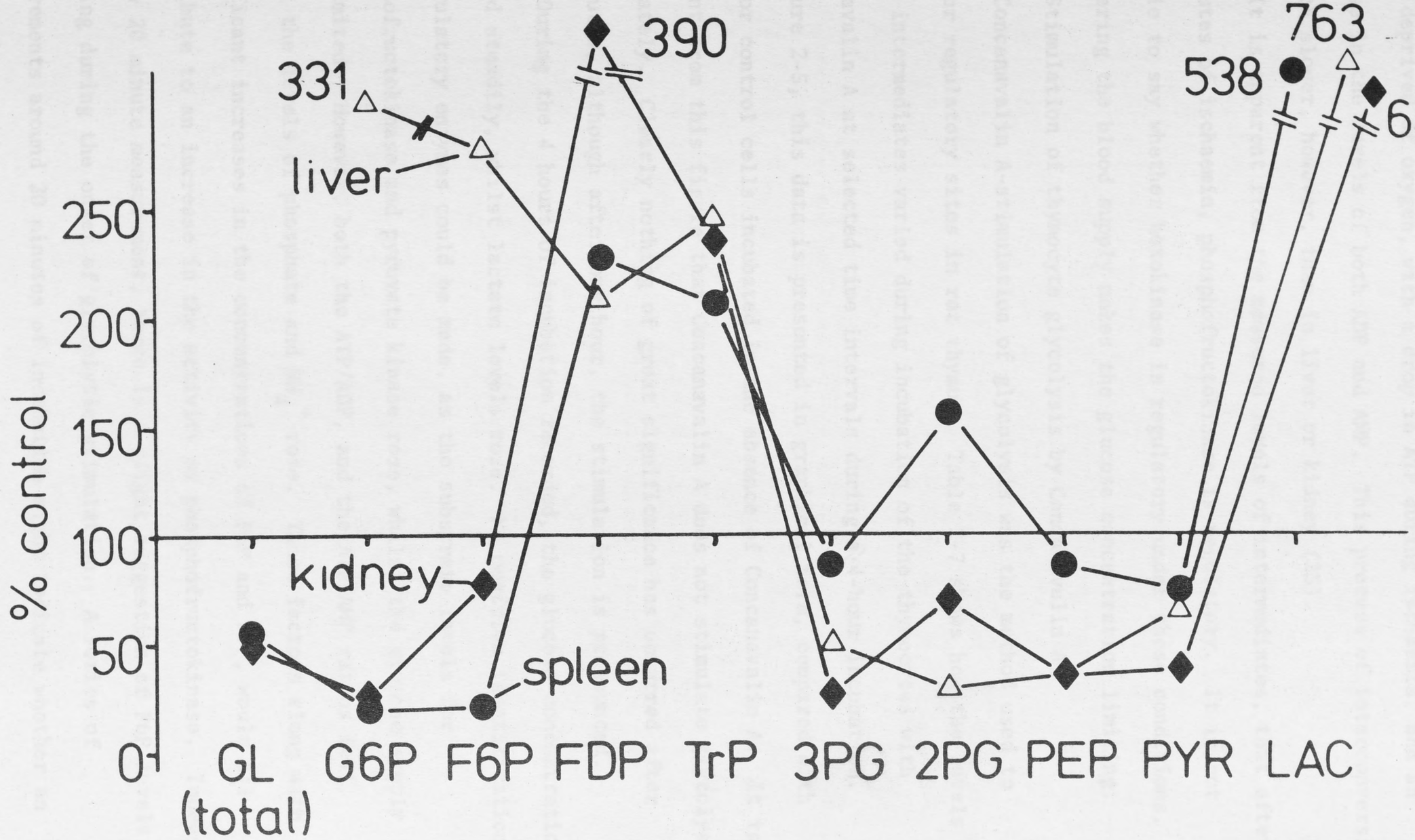
with total flux (as judged by lactate levels) up by at least 600% in 2 minutes. By comparison, it took 10 minutes of ischaemia for lactate levels in rat spleen to increase by 500%. This data and that of Hems and Brosnan (25) for comparative purposes, are presented as a cross-over diagram in Figure 2-4.

During the first 5 minutes of ischaemia, metabolic changes were small. Lactate production increased by less than 100%, and this could be accounted for by the decrease in glucose. Glycogen levels did not change significantly during this period. The second 5 minutes of ischaemia produced an enormous surge of lactate formation, which was, however, unaccompanied by an equivalent change in either glucose or glycogen levels. The last period examined, that between 10 and 15 minutes of ischaemia, found little change in either lactate or glucose levels, but was accompanied by a large disappearance of glycogen. It should be noted that the total amount of carbon in the glucose + glycogen + lactate pool had not significantly changed after 15 minutes of ischaemia, when compared with the zero time controls.

The apparent lack of synchrony between the production of lactate and the disappearance of glucose and glycogen, can be explained by the very large standard errors found, particularly for lactate production. This would suggest that the onset of ischaemia varied greatly between individual spleens. It was observed that the blood content of ischaemic spleens varied considerably, hence it is reasonable to assume that their oxygen contents varied to a similar extent. Considering the smaller standard errors in the measurements of glycogen content, it is reasonable to state that the large surge in glycogen utilization for lactate production which Hems and Brosnan observed at 2 minutes in liver, does not occur for more than 10 minutes in spleen.

Figure 2-4 "Crossover" plot of ischaemic rat spleen, kidney and liver

The levels of glycolytic intermediates in the ischaemic rat spleen, kidney and liver are plotted as a percentage of the control (non-ischaemic *in vivo* levels. The levels of the intermediates in the control are expressed as 100%. Values for the 10 minute ischaemic spleen are taken from Table 2-6. Values for the 2 minute ischaemic liver and kidney are taken from Hems and Brosnan (25).


331
390
763
538
646
liver
kidney
spleen
% control
250
200
150
100
50
0
GL (total)
G6P
F6P
FDP
TrP
3PG
2PG
PEP
PYR
LAC

Changes in adenine nucleotide levels were much as expected in a tissue deprived of oxygen, with a drop in ATP during ischaemia, and an increase in the levels of both ADP and AMP. This process of interconversion was much slower, however, than in liver or kidney (25).

It is apparent from the measured levels of intermediates, that after 10 minutes of ischaemia, phosphofructokinase is regulatory. It is not possible to say whether hexokinase is regulatory under these conditions, as severing the blood supply makes the glucose concentration limiting.

(c) Stimulation of thymocyte glycolysis by Concanavalin A

Concanavalin A-stimulation of glycolysis was the method used to look for regulatory sites in rat thymus. Table 2-7 shows how the levels of the intermediates varied during incubation of the thymocytes with Concanavalin A at selected time intervals during a 4-hour incubation. In Figure 2-5, this data is presented in graphical form, compared with data for control cells incubated in the absence of Concanavalin A. It is apparent from this figure that Concanavalin A does not stimulate glycolysis immediately. Clearly nothing of great significance has occurred after 20 minutes, although after one hour, the stimulation is pronounced.

During the 4 hours of incubation recorded, the glucose concentration dropped steadily, whilst lactate levels rose. No *positive* identification of regulatory enzymes could be made, as the substrate levels for phosphofructokinase and pyruvate kinase rose, whilst the glucose supply was limited. However, both the ATP/ADP, and the ATP/AMP ratios fell, whilst the levels of phosphate and $NH_4^+$ rose. These factors along with significant increases in the concentrations of F6P and FDP, would all contribute to an increase in the activity of phosphofructokinase. Indeed at the 20 minute measurement, there is a slight suggestion of F6P levels dropping during the onset of glycolytic stimulation. A series of measurements around 20 minutes of incubation might indicate whether an

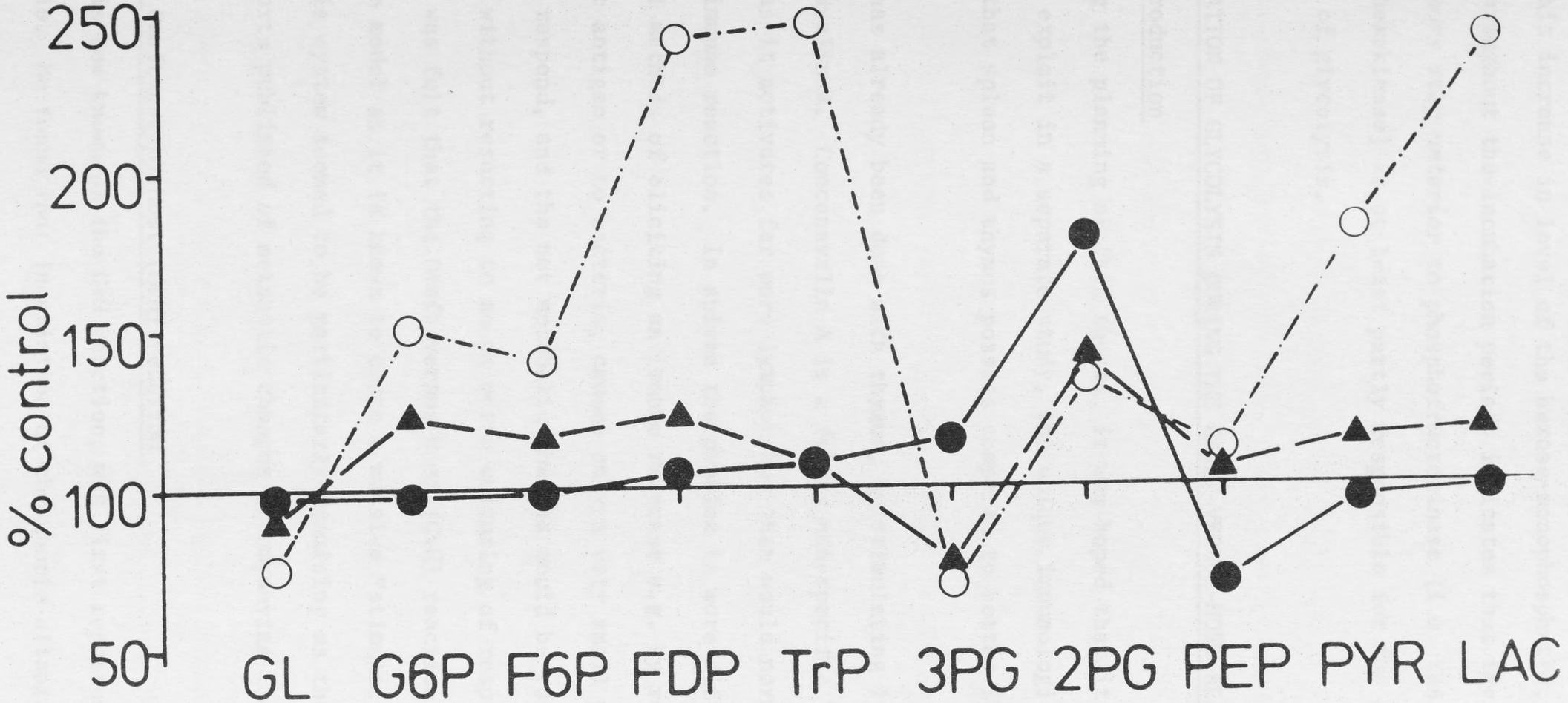
Table 2.7 The concentrations of glycolytic and other intermediates during 4 hours incubation in the presence and absence of Con A

Thymus cells from 32 rats were incubated in the presence and absence of Con A, as described in the text.

| Glycolytic intermediate | Concentration of intermediates (nmoles/$10^{10}$ cells) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Zero time | + 20 minutes | | + 1 hour | | + 4 hours | |
| | | - Con A | + Con A | - Con A | + Con A | - Con A | + Con A |
| Glucose x $10^{-5}$ | 4.84±0.05 | 4.64 (4.75,4.53) | 4.49 (4.17,4.81) | 4.38 (4.28,4.48) | 3.94 (3.88,4.00) | 4.44 (4.32,4.57) | 3.33 (3.39,3.27) |
| Glucose-6-phosphate | 395±5 | 380 (373,386) | 363 (344,383) | 365 (397,332) | 465 (479,451) | 379 (421,338) | 580 (574,587) |
| Fructose-6-phosphate | 110±6 | 114 (113,115) | 110 (105,115) | 121 (102,140) | 141 (137,145) | 126 (144,109) | 179 (179,180) |
| Fructose-1,6-diphosphate | 161±10 | 173 (176,169) | 181 (180,183) | 167 (169,165) | 205 (193,216) | 117 (112,121) | 283 (297,268) |
| Triose-phosphates | 308±17 | 412 (420,413) | 440 (453,427) | 562 (596,528) | 599 (561,636) | 799 (867,731) | 1973 (1881,2064) |
| 3-phosphoglycerate | 64.2±3.1 | 74.5 (74.5,n.d.) | 88.2 (90.1,86.3) | 102 (82.4,122) | 82.3 (82.3,n.d.) | 304 (271,337) | 218 (243,192) |
| 2-phosphoglycerate | 49.3±6.4 | 26.0 (24.0,28.0) | 46.0 (36.0,56.0) | 44.0 (48.0,40.0) | 62.0 (60.0,64.0) | 34.7 (44.0,25.3) | 46.0 (44.0,48.0) |
| Phosphoenolpyruvate | 41.0±3.4 | 50.0 (56.0,44.0) | 34.0 (32.0,36.0) | 60.0 (68.0,52.0) | 62.0 (68.0,56.0) | 103 (103,104) | 111 (98.7,123) |
| Pyruvate | 5458±230 | 5918 (5891,5945) | 5733 (5383,6083) | 5658 (6139,5177) | 6540 (7580,5500) | 2949 (3213,2684) | 5335 (5603,5067) |
| Lactate | 93700±1290 | 107000 (105000,109000) | 105000 (102000,107000) | 121000 (125000,118000) | 146000 (145000,148000) | 83000 (84000,81000) | 199000 (195000,203000) |
| ATP | 3721±178 | 3900 (3913,3887) | 3486 (3188,3784) | 3741 (3907,3576) | 3635 (3693,3576) | 2734 (3110,2358) | 3214 (3602,2825) |
| ADP | 737±31 | 617 (595,639) | 595 (519,671) | 589 (595,583) | 656 (652,660) | 643 (665,622) | 614 (576,652) |
| AMP | 152±2 | 115 (115,115) | 198 (262,134) | 144 (134,153) | 171 (175,167) | 102 (99.5,105) | 253 (239,267) |

Figure 2-5 "Crossover" plot for thymocytes incubated with Con A for 20 minutes ( —●— ), 1 hour (- - ▲ - -) and 4 hours (- · —○· · -)

Values were calculated from the data in Table 2-7, and have been expressed as percentages of the values for controls at the same sampling times.


250
200
150
100
50
% control
GL
G6P
F6P
FDP
TrP
3PG
2PG
PEP
PYR
LAC

early activation of phosphofructokinase precedes the later rise in G6P and F6P. This increase in level of the hexose-monophosphates, which is sustained throughout the incubation period, indicates that the activation of a regulatory step *anterior* to phosphofructokinase (i.e. the glucose carrier or hexokinase) is at least partly responsible for the observed stimulation of glycolysis.

## 2.5 REGULATION OF GLYCOLYSIS DURING THE GRAFT-VERSUS-HOST REACTION

### 2.5.1 Introduction

During the planning of this thesis, it was hoped that it would be possible to exploit in a separate study, the unique immunological properties that spleen and thymus possess compared to better studied organs.

This has already been done with thymus, by stimulating lymphocytes with Concanavalin A. Concanavalin A is a fairly non-specific "antigen analogue", as it activates far more lymphocytes than would normally take part in an immune reaction. In spleen the problem is more difficult, as conventional methods of eliciting an immune response e.g. by reaction to an authentic antigen or to bacteria, causes only a very small population of cells to respond, and the net metabolic changes could be very difficult to monitor, without resorting to an *in vitro* culturing of responsive cells. However, it was felt that the Graft-versus-Host (GvH) reaction might be an effective model as it is known to cause a massive "stimulation" of the spleen. This system seemed to be particularly promising as there have been no reports published of metabolic changes accompanying the GvH reaction.

### 2.5.2 The Graft-versus-Host (GvH) reaction

What is now known as the GvH reaction, was first reported by Murphy (140) in 1916. He found that inoculation of the chorio-allantoic membrane

of chicken embryos, with fragments of spleen or bone marrow from adult chickens, resulted in a considerable enlargement of the host's spleen, and the development of white nodules on the surface of the spleen and other tissues. Examination showed these nodules to be filled with leukocytes. Tissue from adult donors of xenogeneic species failed to cause these systematic changes. Murphy failed to recognize the significance of his results and attributed them to stimulation of the host's spleen. Subsequent work has shown that the following prerequisites must be met before a GvH reaction can occur:

(i) the graft must contain immuno-competent cells;

(ii) the host must possess certain transplantation antigens that the graft lacks, thus making the host "foreign", and enabling the graft to attack it; and

(iii) the host must be incapable of mounting an immune attack against the graft, at least in the early stages of the reaction.

Clearly the genetics of the GvH system are complex. It must be remembered that a GvH reaction will occur only when allogeneic lymphocytes (i.e. lymphocytes from an animal of the same strain, but slightly different genetic constitution) are used. Xenogeneic lymphocytes (where the donor and host animals are unrelated) do not produce a GvH reaction. Bach *et al* (141) working with mice, have shown that the important genetic difference which enables a GvH reaction to occur, is located in only one small sector of the H-2 histocompatibility locus.

A rat suffering from GvH disease displays certain typical clinical and pathological symptoms. These include a falling in body temperature of 2°, a failure to gain weight, and a thickening or loss of skin elasticity. Death typically occurs after about 18 days. Development of GvH disease is usually accompanied by a striking enlargement of the spleen.

Histological examination shows an abnormal and marked proliferation of blast cells and macrophages in the red pulp.

GvH reactions are usually initiated in one of two ways:

(i) the host is immuno-incompetent (e.g. new-born) so that its lymphoid system will not react against the graft; or

(ii) the graft and the host are of such a genetic constitution that the host is foreign to the graft but not vice-versa. Such a case would occur when allogeneic lymphocytes from a homozygote animal (AA) are injected into a host which is a first-generation hybrid (F1, AxB) of the donor strain and a different homozygote (BB). Under these conditions, the donor (AA) will not possess foreign antigens to stimulate the host's (AxB) lymphoid system. The host does, however, contain foreign antigens, and the injected lymphocytes will react against them.

The massive effect on the host's spleen, suggested that the GvH reaction may be a suitable means of producing flux changes in glycolysis in this tissue, allied to a specific immunological event. The remainder of this section describes how the GvH reaction was used in an attempt to identify regulatory sites of glycolysis in rat spleen.

### 2.5.3 Methods

It was decided to initiate the GvH reaction by injecting lymphocytes from a rat of homozygous strain, into a rat which was an F1 hybrid of the donor strain and a different heterozygote.

Most of the methods used have already been described in Section 2.3 above. The following, however, are unique to this section.

(a) Preparation of donor lymphocytes

Donor lymphocyte suspensions were obtained by removing spleens from 10 - 12 week old donor rats (of either Hooded Wistar (HW) or DA strains), cutting these spleens into sections, and then rubbing them against a nylon mesh (500μ) partially immersed in physiological saline, until the residual tissue was white in colour. Large tissue fragments were allowed to settle for 2 - 3 minutes, then the lymphocyte-containing supernatant was decanted into centrifuge tubes, and centrifuged for 5 minutes at 400 x g to pellet the cells. The supernatant was poured off, and 2 mls of water added to lyse the erythrocytes. After mixing the cells for 10 - 15 seconds, an equal volume of double-strength saline was added to restore the original tonicity. The cell suspension was then centrifuged for 5 minutes at 400 x g to pellet the white blood cells. The red cell debris was decanted off and the cell pellets pooled and made up to a concentration of $2 \times 10^8$/ml with physiological saline.

(b) Injection of F1 rats

Male F1 (HWxDA) rats (8 - 10 weeks old) were injected in the tail vein with $4 \times 10^8$ lymphocytes, whilst under ether anaesthesia. They were killed by cervical dislocation at appropriate times after injection, and the spleens and carcasses weighed separately to enable calculation of the body weight/spleen weight ratio.

(c) Evidence for the occurrence of the GvH reaction

One of the more striking changes in animals undergoing GvH reactions, is a gross enlargement of the spleen. This can be quantified as a decreased body weight/spleen weight ratio, and has become accepted as positive evidence for the occurrence of a GvH reaction (142). It is the criterion we have used throughout this study.

2.5.4 Results

(a) Choice of donor

It was decided to use the first generation hybrid of an HWxDA cross as the host. The problem then was which parent homozygote to use for providing the "graft" lymphocytes. Figure 2-6 compares the efficiency of both parents in inducing GvH reactions in the F1 animals, at different times after the donor lymphocytes were injected. Both HW and DA cells lowered the body weight/spleen weight ratio after only 15 - 20 hours. However, the decreased ratio at this stage was probably due to the physical sequestering of foreign cells by the spleen and the entry of large numbers of blood phagocytes. Six-seven days after injection of HW lymphocytes, the ratio had dropped by 30 - 40%, and this was interpreted as evidence of a successfully induced GvH reaction. In contrast, DA lymphocytes gave only an 11% decrease in ratio after 7 days. The very large standard error in the body weight/spleen weight ratio, was a further indication that DA lymphocytes did not induce strong and reproducible GvH reactions. Hooded Wistars were used as lymphocyte donors in all subsequent experiments.

(b) Maximal enzyme activities *in vitro*

The activities of the individual glycolytic enzymes in F1 spleens are very similar to those reported earlier for outbred albino Wistars. Table 2-8 records the activities of the enzymes from F1 spleens, both in normal tissue and in tissue undergoing a GvH reaction. The activities in these two groups are also very similar. As in the spleens of albino Wistars, the low activity enzymes are hexokinase, phosphofructokinase, aldolase and glucose-6-phosphate dehydrogenase.

(c) Identification of non-equilibrium reactions

Table 2-9 shows the mass-action ratios and the apparent equilibrium constants for the individual reactions of glycolysis in freeze-clamped F1

Figure 2-6 Effect of time and lymphocyte source on the body weight/spleen weight ratio during a GvH reaction

F1 (HWxDA) rats were injected with $4 \times 10^8$ lymphocytes from the spleens of either DA or HW donors. Body weight/spleen weight ratios are plotted as a percentage of the control ratios (obtained from uninjected animals) at different times after injection and the results are the means ± S.E.M.

(—●—) Hooded Wistar; (—△—) D.A.

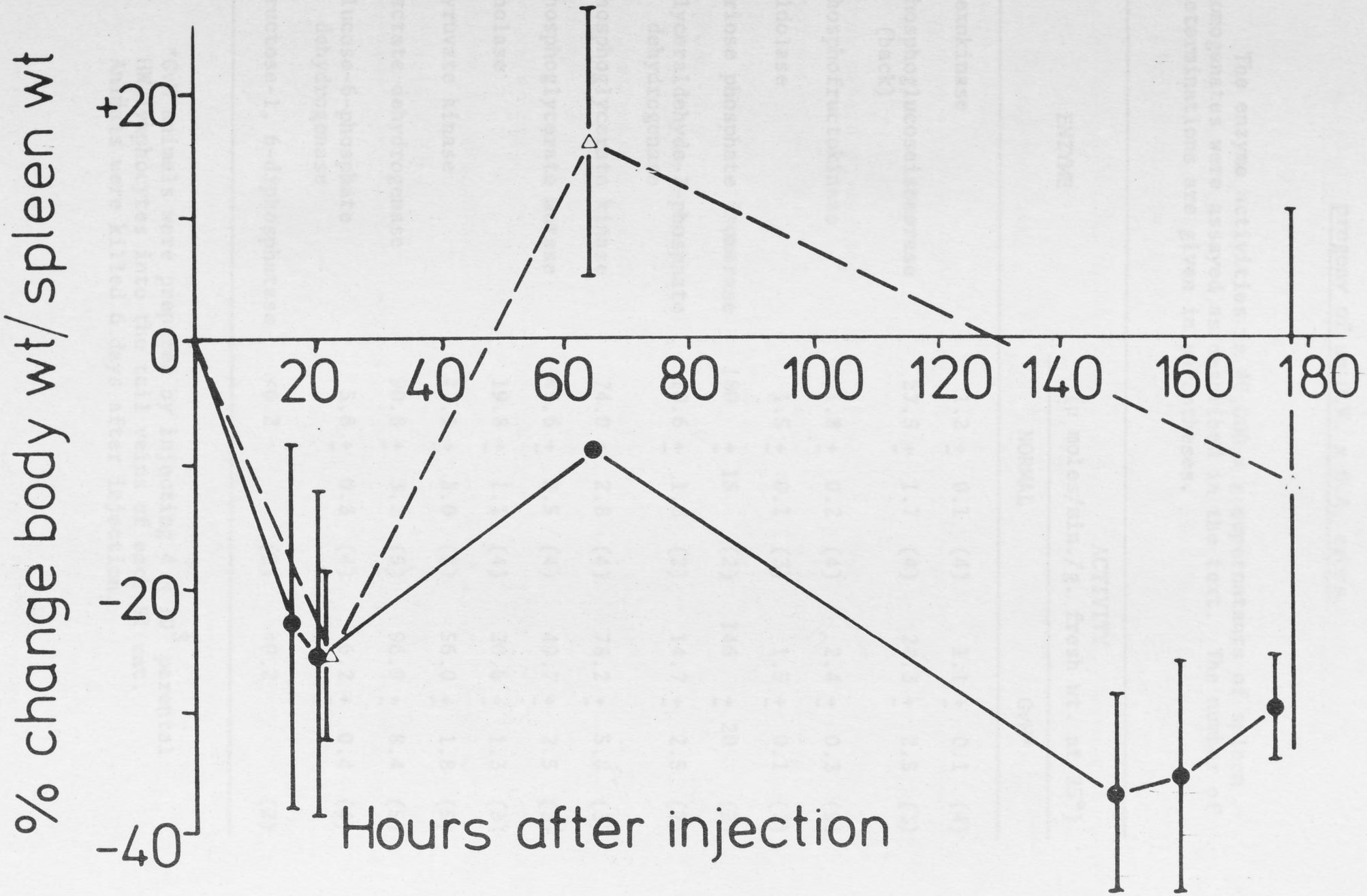

% change body wt/ spleen wt
+20
0
-20
-40
20
40
60
80
100
120
140
160
180
Hours after injection

Table 2-8

Glycolytic enzyme activities in the spleen of inbred male F1 rats, progeny of an H.W. x D.A. cross

The enzyme activities in 40,000 x g supernatants of spleen homogenates were assayed as described in the text. The number of determinations are given in parentheses.

| ENZYME | ACTIVITY (μ moles/min./g. fresh wt. at 25°) | | | | | |
|---|---|---|---|---|---|---|
| | NORMAL | | | GvH* | | |
| Hexokinase | 1.2 | ± 0.1 | (4) | 1.1 | ± 0.1 | (4) |
| Phosphoglucoseisomerase (back) | 27.5 | ± 1.7 | (4) | 24.3 | ± 2.5 | (3) |
| Phosphofructokinase | 1.8 | ± 0.2 | (4) | 2.4 | ± 0.3 | (5) |
| Aldolase | 1.5 | ± 0.1 | (3) | 1.5 | ± 0.1 | (3) |
| Triose phosphate isomerase | 180 | ± 15 | (2) | 146 | ± 20 | (2) |
| Glyceraldehyde-3-phosphate dehydrogenase | 10.6 | ± 1.3 | (2) | 14.7 | ± 2.5 | (2) |
| Phosphoglycerate kinase | 74.0 | ± 2.8 | (4) | 78.2 | ± 5.8 | (3) |
| Phosphoglycerate mutase | 45.6 | ± 2.5 | (4) | 49.7 | ± 2.5 | (3) |
| Enolase | 19.8 | ± 1.1 | (4) | 20.6 | ± 1.3 | (3) |
| Pyruvate kinase | 47.1 | ± 3.0 | (5) | 56.0 | ± 1.8 | (5) |
| Lactate dehydrogenase | 90.8 | ± 3.2 | (5) | 96.9 | ± 8.4 | (5) |
| Glucose-6-phosphate dehydrogenase | 5.8 | ± 0.3 | (4) | 6.2 | ± 0.4 | (4) |
| Fructose-1, 6-diphosphatase | <0.2 | | (2) | <0.2 | | (2) |

*GvH animals were prepared by injecting $4 \times 10^8$ parental HW lymphocytes into the tail veins of each F1 rat. Animals were killed 6 days after injection.

Table 2-9

Mass-action ratios versus equilibrium constants for the glycolytic pathway in F1 (D.A. x H.W.) freeze-clamped spleens

Mass-action ratios were calculated from the concentrations of intermediates given in Table 2-10. Concentrations given as n. moles/g. fresh wt. were taken as equivalent to μM. The remaining details of calculation were as for Table 2-5.

| ENZYME REACTION | APPARENT EQUILIBRIUM CONSTANT (K') | MASS-ACTION RATIO (Γ) | RATIO Γ/K' |
|---|---|---|---|
| Hexokinase | 4700 | $1.1 \times 10^{-2}$ | $2.34 \times 10^{-6}$ |
| Phosphoglucoseisomerase | 0.41 | $2.8 \times 10^{-1}$ | 0.68 |
| Phosphofructokinase | 1003 | $4.2 \times 10^{-1}$ | $4.2 \times 10^{-4}$ |
| Aldolase | $9.6 \times 10^{-5}$ | $5.4 \times 10^{-6}$ M | $5.6 \times 10^{-2}$ |
| G-3P-dh plus phosphoglycerate kinase | 59 | 92 | 1.56 |
| Phosphoglycerate mutase | 0.15 | $3.1 \times 10^{-1}$ | 2.06 |
| Enolase | 2.93 | $7.5 \times 10^{-1}$ | 0.26 |
| Pyruvate kinase | 17,900 | 16.1 | $9 \times 10^{-4}$ |
| Lactate dehydrogenase | assumed at equilibrium | | |
| Adenylate kinase | 0.44 | 0.31 | 0.72 |

spleens. As with the albino Wistar spleens, phosphofructokinase, aldolase and pyruvate kinase all catalyze non-equilibrium reactions. Although no attempt has been made to differentiate between intra- and extra-cellular glucose, the results indicate, by analogy with the results from albino Wistar spleens, that hexokinase also catalyzes a non-equilibrium reaction in F1 spleen.

(d) Identification of regulatory enzymes using the GvH reaction

The levels of glycolytic and other intermediates in the freeze-clamped spleens from both normal F1 rats and F1 rats undergoing a GvH reaction, are presented in Table 2-10. Although the body weight/spleen weight ratio had decreased by 40% after 6 days GvH, there is no indication of a change in glycolytic flux rate. There is a suggestion that glucose levels have fallen, but the difference is not significant. Changes in the glycolytic intermediates are presented as a cross-over diagram in Figure 2-7. The concentrations of both hexose-monophosphates have increased significantly, whilst the concentrations of FDP and the phosphoglycerates have fallen significantly. This data suggests a decreased flux through phosphofructokinase.

Changes in the levels of non-glycolytic intermediates agree with an inhibition of phosphofructokinase. ATP levels rose, and ADP levels fell, whilst both the ATP/ADP and ATP/ADPxPi ratios increased. In view of the extreme sensitivity of phosphofructokinase to changes in these ratios, an inhibition is to be expected. It should be noted that the concentration of the potent stimulator AMP, increased under the same conditions. However, spleen phosphofructokinase has an apparent $K_A$ for AMP much lower than the levels observed here (see Chapter 3), so it is probable that the change had little effect.

The data presented above is somewhat contradictory, in that although there appears to be decreased flux through phosphofructokinase, the net

Table 2-10

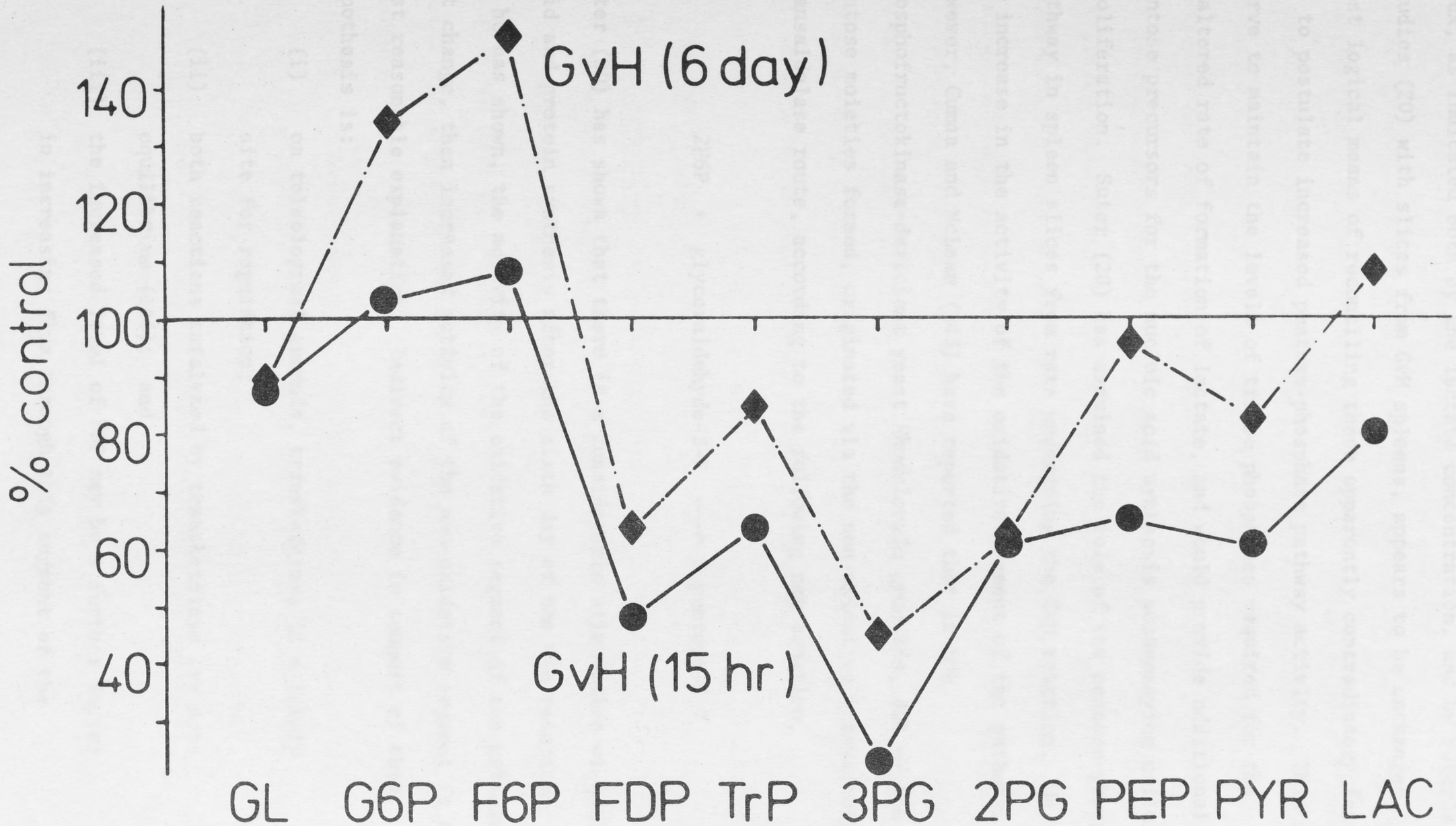
Levels of glycolytic and related intermediates in freeze-clamped spleens from normal and GvH F1 rats

The intermediates were assayed in extracts prepared as described in the text. The concentrations are expressed as n. moles/g. fresh wt. All results are the means ± S.E.M. of 4 or 5 values as indicated in parentheses. Where results have been tested for statistical significance: N.S., means not statistically significant.

| PARAMETER MEASURED | CONTROL (5) (uninjected) | GvH (5) (15 hr.) | GvH (4) (6 day) | |
|---|---|---|---|---|
| Body wt./spleen wt. | 459 ± 15 | 351 ± 36 | 286 ± 20 | |
| Glucose | 2206 ± 149 | 1955 ± 53 | 1924 ± 97 | |
| G-6-P | 84 ± 6 | 87 ± 6 | 113 ± 8 | Sig. P <5% |
| F-6-P | 24 ± 2 | 26 ± 2 | 36 ± 3 | Sig. P <2% |
| FDP | 35 ± 4 | 17 ± 2 | 22 ± 1 | Sig. P <5% |
| Triose-P | 76 ± 10 | 49 ± 6 | 65 ± 12 | N.S. |
| 3-PGA | 90 ± 4 | 24 ± 5 | 37 ± 2 | Sig. <0.1% |
| 2-PGA | 28 ± 4 | 17 ± 3 | 17 ± 3 | P <5% |
| PEP | 21 ± 3 | 14 ± 1 | 20 ± 3 | N.S. |
| Pyruvate | 94 ± 8 | 59 ± 3 | 79 ± 6 | N.S. |
| Lactate | 1267 ± 113 | 1004 ± 107 | 1388 ± 186 | N.S. |
| Lac/Pyr | 13.6 ± 1.2 | 17.3 ± 2.3 | 22.2 ± 1.9 | |
| ATP | 2565 ± 78 | 2670 ± 144 | 2655 ± 69 | |
| ADP | 718 ± 37 | 639 ± 42 | 606 ± 28 | |
| AMP | 63 ± 3 | 53 ± 4 | 84 ± 7 | |
| Pi | 2614 ± 62 | 2633 ± 186 | 2622 ± 98 | |
| ATP/ADP | 3.60 ± 0.17 | 4.20 ± 0.09 | 4.40 ± 0.16 | |
| Energy Charge | 0.87 ± 0.004 | 0.89 ± 0.002 | 0.89 ± 0.006 | |
| ATP/ADP x Pi (measured) | 2303 ± 128 | 2725 ± 248 | 2807 ± 147 | |

Figure 2-7 "Crossover" plot during a GvH reaction

The levels of glycolytic intermediates in the 15 hour and 6 day GvH spleens are plotted as a percentage of the control. The levels of the intermediates in the control are expressed as 100% and all the values are taken from Table 2-10.


GvH (6 day)
GvH (15 hr)
% control
140
120
100
80
60
40
GL
G6P
F6P
FDP
TrP
3PG
2PG
PEP
PYR
LAC

flux, as indicated both by the lactate concentration, and by Suter's studies (20) with slices from GvH spleens, appears to be unchanged. The most logical means of reconciling these apparently contradictory facts, is to postulate increased pentose-phosphate pathway activity. This would serve to maintain the levels of triose phosphates required for the unaltered rate of formation of lactate, and would provide additionally, pentose precursors for the nucleic acid synthesis accompanying cellular proliferation. Suter (20) has examined the role of the pentose-phosphate pathway in spleen slices from rats undergoing the GvH reaction. He found no increase in the activity of the oxidative segment of the pathway. However, Gumaa and McLean (143) have reported that in the phosphofructokinase-deficient yeast *Rhodolorula gracilis*, 80% of the pentose moieties formed, originated via the non-oxidative transketolase-transaldolase route, according to the following net equation,

$$2F6P + \text{glyceraldehyde-3-P} \longrightarrow 3 \text{ pentose-5-P}$$

Suter (20) has shown that there is a considerable stimulation of nucleic acid and protein synthesis after the sixth day of the GvH reaction. If, as he has shown, the activity of the oxidative segment of the pathway does not change, then increased activity of the non-oxidative segment is the most reasonable explanation. Indirect evidence in support of this hypothesis is:

(i) on teleological grounds, transketolase is a likely site for regulation;

(ii) both reactions catalyzed by transketolase are non-equilibrium (143); and

(iii) the increased level of F6P may be a further factor in increasing flux through this segment of the

pentose-phosphate pathway.

It is unfortunate that the results presented in this work are not more clear cut. However, this project is concerned with the identification of regulatory enzymes, not with the quantitative aspects of glucose metabolism. The results presented in this section *do* suggest that glycolysis has been inhibited at the locus of phosphofructokinase, and that increased pentose-phosphate pathway activity may be responsible for maintaining lactate at a level similar to that of the controls. More recent data from Weidemann (121) suggests that the sequence of metabolic events involved in lymphocyte transformation may involve *multiple* control points, and that the apparent inhibition of phosphofructokinase observed here may be genuine. These results will be discussed more fully in Chapter 4, where a consideration of the role of glycolysis in the initiation of lymphocyte transformation is presented.

## 2.6 ENZYME STUDIES

### 2.6.1 Introduction

The results described in the earlier part of this chapter, have shown that phosphofructokinase can nearly always be identified as a step which controls the rate of flux through the glycolytic pathway in lymphoid tissue. To complete this section of the work, two small studies were undertaken:

(i) a brief examination of the properties of rat spleen phosphofructokinase in a crude supernatant preparation, to see whether it displayed any of the properties which a regulatory enzyme might be expected to possess, and

(ii) an investigation of the isoenzymes of spleen pyruvate kinase and lactate dehydrogenase.

One problem which was still of some concern, was the question of which cell types were responsible for the observed metabolic activity. Spleen comprises three major cell types, lymphocytes, erythrocytes and phagocytes. It was hoped that by determining the isoenzyme spectrum for these two enzymes it would be possible to say with more precision which of the three cell types contributed most to the observed activity patterns.

2.6.2 Phosphofructokinase

Rat spleen phosphofructokinase was prepared by homogenizing tissue in 3 volumes of a buffer containing 50 mM Triethanolamine-HCl, pH 7.5, containing 1 mM EDTA. The resultant suspension was centrifuged twice, — for 15 minutes at 20,000 x g to remove mitochondria, and for 60 minutes at 100,000 x g to settle all microsomes. All assays were conducted at pH 7.0 in the Imidazole buffer used by Underwood and Newsholme (52). The following properties were observed:

(i) in the presence of only 0.7 mM ATP, titration of phosphofructokinase activity with F6P produced a strongly sigmoidal curve with a Hill coefficient of 1.6;

(ii) in the presence of 0.7 mM ATP, AMP (0.5 mM) stimulated phosphofructokinase activity nearly 3-fold when F6P levels were low (0.1 mM). If, however, the F6P concentration was raised to 0.3 mM, AMP caused little activation, and

(iii) under similar conditions to (ii) above, both ADP and cAMP were also stimulatory, ADP only slightly, but cAMP to nearly the same extent as AMP.

A sigmoidal response to one of its substrates, and activation or inhibition by some molecule which is not a substrate for the reaction it catalyzes, are two properties which a regulatory enzyme typically possesses.

This confirmation of phosphofructokinase's regulatory capacity leads to Chapter 3 of this thesis, which presents an in-depth examination of the kinetic properties of a partially-purified phosphofructokinase from spleen.

2.6.3 Isoenzyme studies

(a) Pyruvate Kinase

The most obvious differentiation of cell types in spleen, is into erythrocytes and leukocytes. Suter (20) has found that rat spleen contains approximately equal proportions of each cell type. Pyruvate kinase exists in two enzymatic forms, liver (L)-type and muscle (M)-type. The L-isoenzyme is activated by the allosteric effector FDP and is the only form found in erythrocytes, whilst the M-isoenzyme is not activated by FDP and is the only type present in leukocytes (144). Thus by determining which isoenzyme is predominant in spleen extracts, it is possible to say which cell type contributes most to the observed metabolic activity.

Spleen pyruvate kinase was assayed for activation by FDP, by the method of Taylor and Bailey (145). FDP had no effect at all on pyruvate kinase kinetics, implying that erythrocytes contribute very little, if at all, to the metabolic activity in rat spleen.

(b) Lactate dehydrogenase

There are two basically different forms of lactate dehydrogenase in animal tissues. The form which predominates in heart is composed of four identical subunits and is called the H enzyme. The form which predominates in skeletal muscle is also composed of identical subunits different from those in heart and is called the M enzyme. These two forms of lactate dehydrogenase exist to different extents in the various tissues of the body, and it is possible to have any of five different isoenzymes present, of form $H_4$, $H_3M_1$, $H_2M_2$, $H_1M_3$ and $M_4$.

The H enzyme is strongly inhibited by concentrations of pyruvate greater than 1 mM, whilst the M enzyme is relatively insensitive to inhibition by its substrate. By comparing the activity of lactate dehydrogenase in the presence of 0.3 mM pyruvate, to that in 1.0 mM pyruvate, it is possible to make an approximate assessment of the ratio of H and M subunits of the enzyme present (146). For spleen lactate dehydrogenase, the ratio $\frac{\text{activity in 0.3 mM pyruvate}}{\text{activity in 10 mM pyruvate}}$ is 1.4, whilst for the thymus enzyme the value is 1.8. By reference to the results of Wilson *et al* (146) this suggests that spleen lactate dehydrogenase is composed of approximately 40% H subunits, whilst thymus lactate dehydrogenase comprises approximately 50% H subunits. Thymus contains an almost pure population of lymphocytes, so it is probable that thymocytes contain approximately equal mixtures of H and M subunits. Granulocytes and other phagocytic cells are reported to contain a preponderance of M subunits (147). Thus it seems probable that the glucose metabolism of spleen is due *largely* to the lymphocytes within it, though with a definite contribution from the phagocytic cells. Suter (20) has reached the same general conclusion in his studies on the quantitative aspects of glucose metabolism in spleen slices.

## 2.7 DISCUSSION

The aim of the work reported in this chapter, was to determine, in accordance with the principles established by Newsholme and Gevers (13), which enzymes regulate glycolytic flux in the two major mammalian lymphoid tissues, spleen and thymus.

Freeze-clamping of both tissues, and subsequent determination of the levels of glycolytic intermediates, provides an experimental basis for identifying which metabolic reactions are significantly removed from equilibrium, as it is the enzymes catalyzing these reactions which may

possess regulatory properties. As a refinement, extracellular spaces were calculated, allowing separate estimations of the equilibrium positions of the glucose carrier and hexokinase to be made. It was concluded that hexokinase, phosphofructokinase and pyruvate kinase, catalyze reactions removed far from equilibrium, whilst the glucose carrier and aldolase catalyze reactions which are "intermediate" between the equilibrium and non-equilibrium states.

When glycolysis was varied experimentally in lymphoid tissues, it became apparent that two subtle though distinctly different patterns emerged, depending on the means utilized to perturb flux. Utilizing Krebs' principle (18) that a change in substrate concentration of a non-equilibrium enzyme in the opposite direction to the change in net flux is indicative of an enzyme being regulatory, it is clear that phosphofructokinase meets this criterion during both ether anaesthesia and anoxia. A drop in total glucose concentration indicated that the glucose carrier and/or hexokinase were regulatory during anaesthesia. It is not possible to positively ascribe any regulatory role to either the glucose carrier or hexokinase during anoxia, as severance of the blood supply and isolation of the organ made the glucose supply limited. The metabolic pattern observed during anoxia is very similar to that seen in other tissues; indeed, as can be seen in Figure 2-4, the pattern for the ten minute anoxic spleen can almost be superimposed upon that of the two minute anoxic kidney. In situations where changes in metabolic flux are induced by challenging the *integrity* of the tissue's respiratory system, the term *emergency glycolysis* is used. In the present work where such a stress has been applied (i.e. anoxia, ether anaesthesia), it is clear that phosphofructokinase is primarily responsible for regulating flux through the pathway, whilst the glucose carrier and/or hexokinase may also be involved, either directly (e.g. by activation of the carrier) or indirectly

(e.g. by deinhibition), following intermediate changes brought about by the activation of phosphofructokinase.

The other pattern of glycolysis observed, is that which occurs when the organ's immunological defences are challenged. For convenience, the term *immunological glycolysis* is used. Two examples of this condition are offered, — the Con A-activation of thymocytes, and the GvH reaction in rat spleen. During the GvH reaction, the most note-worthy events are the reduction in glucose concentration, the increase in hexose-monophosphate concentration, and the apparent constancy of flux. During the Con A-activation of thymocytes, the levels of the hexose-monophosphates increase steadily in the same direction as the flux rate, whilst the levels of FDP and triose-phosphates increase later, but to a much greater extent. The only regulatory step which can be *positively* identified during either of these processes is the glucose carrier and/or hexokinase during the GvH reaction. Although phosphofructokinase cannot be positively identified as regulatory according to the criteria employed above, changes in the levels of its effectors are consistent with it being regulatory under both conditions: inhibited during the GvH reaction and activated during Con A stimulation.

On first inspection it appears unreasonable to compare Con A-activation and the GvH reaction, as one demonstrates a strong activation of glycolysis, whilst the other, if not inhibition, at least indicates no change. However, it must be remembered that Con A is a non-specific activator which affects a far larger population of lymphocytes (~50%) than those normally taking part in a true immunological reaction (<5%). The GvH reaction on the other hand is highly specific, immunologically, and would not necessarily exhibit an identical metabolic pattern. What is of prime importance in both cases, is the *increased* concentration of hexose-monophosphates. Weidemann and Kolbuch (121) have recently produced

evidence which suggests that significant glycogen synthesis may occur during the first 3 hours of Con A stimulation. The appropriate results will be quoted in Chapter 4, where an attempt will be made to define the role of glycolysis during lymphocyte activation. If, as Weidemann suggests (121), glycogen storage is a feature of the early events associated with glucose uptake then, clearly, an initial activation of glucose transport and/or hexokinase, may produce the raised level of G6P necessary to direct glucose carbon to glycogen. The apparently inconsistent data relating to phosphofructokinase, i.e. apparent activation with Con A, and apparent inhibition during the GvH reaction, can be reconciled with this data. The GvH reaction is a very specific immunological reaction, and furthermore, the measurements reported here were carried out at time intervals much later than 3 hours after initiation of the reaction. All the measurements on the Con A-activation of thymocytes were carried out at time intervals less than 4 hours after mitogen addition. When the events observed after long term incubation with mitogen are considered, then the two sets of data become reconcilable. Glycolytic flux rate is reported to be elevated between 30 minutes and 8 hours after mitogen addition (115), whilst RNA and protein synthesis and DNA synthesis are not activated till approximately 24 (118) and 60 hours (118) respectively. Thus although during the GvH reaction the period of glycolytic stimulation is over, pentose moieties are still required for the subsequent synthetic events. This will explain in turn, the apparently steady state of glycolytic flux, and the increased utilization of glucose and elevated levels of hexose-monophosphates. From the two experiments conducted relating to *immunological glycolysis*, it seems that the prime regulatory event is the activation of glucose transport and/or hexokinase, whilst regulation at the phosphofructokinase locus may be of secondary importance.

One point of interest when comparing the intermediate profiles of spleen and thymus (see Tables 2-3 and 2-4), is the difference in the lactate/pyruvate ratios. In spleen, this value is 10.5, whereas in the thymus it is 23.9. If the tissues are similar in metabolic properties, it is likely that their lactate/pyruvate ratios will be the same. When tissues become anoxic, their lactate/pyruvate ratios increase as the oxidation/reduction state of the cytoplasmic $NAD^+$/NADH ratio is perturbed, and it might seem, at first, that the thymii used in the determination of enzyme equilibrium positions were at least partially anoxic. However, calculations of the energy charge made from adenine nucleotide determinations on the same tissues, gave a value of 0.83, indicative of healthy aerobic organs. In addition, Weidemann (121) has found the lactate/pyruvate ratio in aerobic thymocytes incubated in the presence of glucose and 100% oxygen to be 21.5. Thus, this high ratio appears to be characteristic of the tissue.

The differences in the lactate/pyruvate ratio, appears to be the major difference between the two tissues. The studies reported in Section 2.6 showed differences, but not of any great extent. It must be remembered, that during the GvH reaction spleens nearly doubled in size, and as this is primarily a lymphocyte-proliferative reaction, it is clear that lymphocytes contribute a large proportion of the metabolic activity within this tissue.

The results contained in this Chapter, indicate clearly that both phosphofructokinase and the glucose carrier/hexokinase locus are important regulatory sites in the metabolism of glucose. A preliminary investigation of the properties of phosphofructokinase (see Section 2.6) showed that it possessed properties indicative of a regulatory function. Chapter 3 reports the purification and examination of the properties of phosphofructokinase, whilst the Discussion of that chapter considers

whether the observed properties are compatible with phosphofructokinase regulating glycolysis in lymphoid tissue.

# CHAPTER 3

## THE PURIFICATION AND PROPERTIES OF PIG SPLEEN PHOSPHOFRUCTOKINASE

# CHAPTER 3

# THE PURIFICATION AND PROPERTIES OF PIG SPLEEN PHOSPHOFRUCTOKINASE

## 3.1 INTRODUCTION

The work recorded in Chapter 2 of this thesis, shows that phosphofructokinase is, at least under conditions of oxygen deprivation or ether anaesthesia, the enzyme primarily responsible for controlling glycolytic flux in rat spleen. The next logical step in the investigation should be to purify and make a detailed kinetic study of spleen phosphofructokinase.

Although no results will be presented, a pilot study was conducted with the enzyme from rat spleen. To this end, a partially purified preparation with a Specific Activity of only 3U/mg protein was obtained. However, the physical quantity obtained in a single preparation from 30 spleens was so small that detailed kinetic studies were not feasible.

It was finally decided to purify the enzyme from pig spleen, where starting material was available in kilogram quantities. This chapter, besides recording the purification and some of the kinetic properties of pig spleen phosphofructokinase, also reports a comparison of some of the more significant kinetic properties of the enzyme from pig spleen, rat spleen and rat thymus, and concludes with some experiments simulating the effect of *in vivo* conditions on phosphofructokinase activity.

## 3.2 MATERIALS

All nucleotides and fine chemicals used in this study, were purchased from Boehringer Mannheim. They were either purchased as the $Na^+$-salts, or were converted to this form with $Na^+$-Dowex before use. All other chemicals used were of analytical grade.

The coupling enzymes used, namely aldolase, triose-phosphate isomerase, α-glycerophosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase, were purchased from either Boehringer Mannheim, or the Sigma Chemical Co., St. Louis, Missouri.

Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was purchased from Sigma, and DE 52 DEAE-cellulose from the Whatman Company.

Pig spleens were obtained from the Canberra Abbatoirs. They were placed on ice within 5 minutes of death and transported back to the laboratory in a large Dewar flask. Fat was trimmed off samples, which were then cut into pieces of approximately 10 g weight before being frozen at -20°.

## 3.3 METHODS

### 3.3.1 Enzyme assays

Measurements of phosphofructokinase activity are usually made in either of two pH regions;

(i) pH 8.0 - 8.2, at which activity is maximal, or

(ii) pH 6.8 - 7.2, which is near-physiological, and at which the regulatory properties of the enzyme can be clearly seen.

Ideally the buffer used should have good buffering capacity in both of these pH ranges, i.e. it should have an intermediate pKa. Commonly used buffers such as Tris, glycylglycine and imidazole are effective buffers in only one of these pH regions. Hepes, however, has an intermediate pKa of 7.55, and does not bind metal ions (148), so for this reason it was selected as the assay buffer.

For examination of its regulatory properties, phosphofructokinase was assayed in 50 mM Hepes-NaOH pH 7.0, containing ATP, F6P and effectors as indicated, with 0.1 mM NADH. All solutions of effectors were adjusted

to pH 7.0 with NaOH. $Mg^{2+}$ was always 3 mM in excess of the ATP concentration. Phosphofructokinase activity was measured by the decrease in optical density at 340 nm on a Zeiss PMQII spectrophotometer using aldolase, triose-phosphate isomerase and α-glycerophosphate dehydrogenase, approximately ten times in excess of the observed activity of phosphofructokinase. Coupling enzymes were dialyzed for 24 hr. against 3 one litre volumes of 50 mM Tris-Cl pH 8.0, containing 0.05 mM EDTA, to remove $(NH_4)_2.SO_4$. No $NH_4^+$ could be detected on subsequent enzymatic assay. Immediately before assay, stock solutions of phosphofructokinase were diluted with 80 mM potassium phosphate buffer pH 8.0, containing 1 mM EDTA, until a suitable optical density change could be recorded.

For assays of maximal activity during enzyme purification, phosphofructokinase was assayed in 50 mM Hepes-NaOH pH 8.0, containing 0.4 mM ATP, 2.0 mM F6P, 3.0 mM $Mg^{2+}$ and 0.1 mM NADH. Coupling enzymes were added in approximately tenfold excess, but without prior dialysis.

It is commonly reported in the literature that assays of phosphofructokinase are conducted at a fixed concentration of $Mg^{2+}$. As results presented later in this thesis show, the relative levels of $Mg^{2+}$ and ATP are of considerable importance in determining what the absolute activity of the enzyme will be. Computer calculations (not recorded) using the stability constants of Morrison and Heyde (149) showed that only when $Mg^{2+}$ is present in considerable excess, is the ATP nearly quantitatively bound. For this reason, the practice of Paetkau and Lardy (67) of keeping $Mg^{2+}$ constantly 3 mM in excess over the ATP concentrations has been adopted.

Newsholme *et al* (150) have shown that NADH will inhibit the coupling enzymes used in this assay system. For this reason, the initial level of NADH is kept constant and low at 0.1 mM.

Reported methods of initiating enzyme assays vary. For example, Kemp (54) and Mansour and Ahlfors (65) preincubated phosphofructokinase with ATP for 2 minutes before initiating the reaction with F6P. Other workers (e.g. 11, 57, 60) initiated the reaction with enzyme. In our studies, we have found that phosphofructokinase is rapidly inactivated when preincubated with ATP. Bearing in mind the proposed association/dissociation model for regulation of phosphofructokinase activity (103), it is apparent that preincubating with either substrate at the expense of the other would create a physiologically unnatural state where the enzyme tends to be either far more, or far less aggregated than would be the case *in vivo*. On the other hand, if the reaction is initiated with the enzyme, it will be exposed to all the substrates and effectors simultaneously, and apparently the equilibrium position between active and inactive forms will be reached more rapidly. For this reason, in all the kinetic experiments reported below, reactions were initiated by addition of enzyme.

### 3.3.2 Enzyme purification

Pig spleen phosphofructokinase was purified by a modification of the method of Massey and Deal (159). Full details of the purification procedure are contained in Section 3.4.1 below. Buffers used were as follows:

Buffer A - 50 mM Tris-Cl, 50 mM β-mercaptoethanol and 5 mM EDTA, pH 8.0;

Buffer B - 50 mM Tris-Cl, 50 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.1 mM ATP and 0.1 mM FDP, pH 8.0,

Buffer C - Buffer B + 110 mM $(NH_4)_2.SO_4$; and

Buffer D - 80 mM potassium phosphate and 1 mM EDTA, pH 8.0.

Rat spleen phosphofructokinase was prepared in the same manner as the pig spleen enzyme, as far as the $Mg^{2+}$-precipitation step. Rat

thymocyte phosphofructokinase was prepared essentially as described in Section 2.3.1 above, save that Buffer A for the purification of pig spleen phosphofructokinase was used for homogenization.

### 3.3.3 Protein determination

Protein concentrations were determined by a minor modification of Hartree's method (160). In our system, β-mercaptoethanol gave a large blank which made it difficult to obtain accurate readings. Therefore, as a routine procedure, all protein samples were precipitated prior to assay by adding TCA to a final concentration of 5%. Samples were allowed to stand in the cold for 30 minutes, then the precipitates were collected by centrifugation, and made up to volume. Subsequent steps were as described by Hartree (160).

### 3.3.4 Analytical methods

The concentrations of all substrate and effector molecules were accurately determined by enzymatic assay, using the methods described in Section 2.3.4.

## 3.4 RESULTS

### 3.4.1 Purification of pig spleen phosphofructokinase

Pig spleen phosphofructokinase was partially purified by a modification of the method of Massey and Deal (159). Unless indicated otherwise, all procedures were conducted at 4°.

1. *Homogenization.* Frozen spleens were thawed overnight on ice. They were minced coarsely, then placed in a Waring Blendor with 2 volumes of ice-cold Buffer A. The suspension was then homogenized: once for 30 seconds on "fast", and twice for 40 seconds on "slow". The resulting homogenate was then centrifuged for 40 minutes at 13,000 x g in a Sorvall RC-2B refrigerated centrifuge, and the supernatant removed by decanting.

2. *Heat-ethanol treatment.* To the supernatant fluid, β-mercaptoethanol

(1 ml/100 ml solution) and 95% ethanol (20 ml/100 ml solution) were added. The solution was placed in a water bath at 41° and shaken at 100 oscillations per minute for 45 minutes. At the end of the incubation period the suspension was centrifuged for 15 minutes at 10,000 x g, and the clear red supernatant decanted and stored.

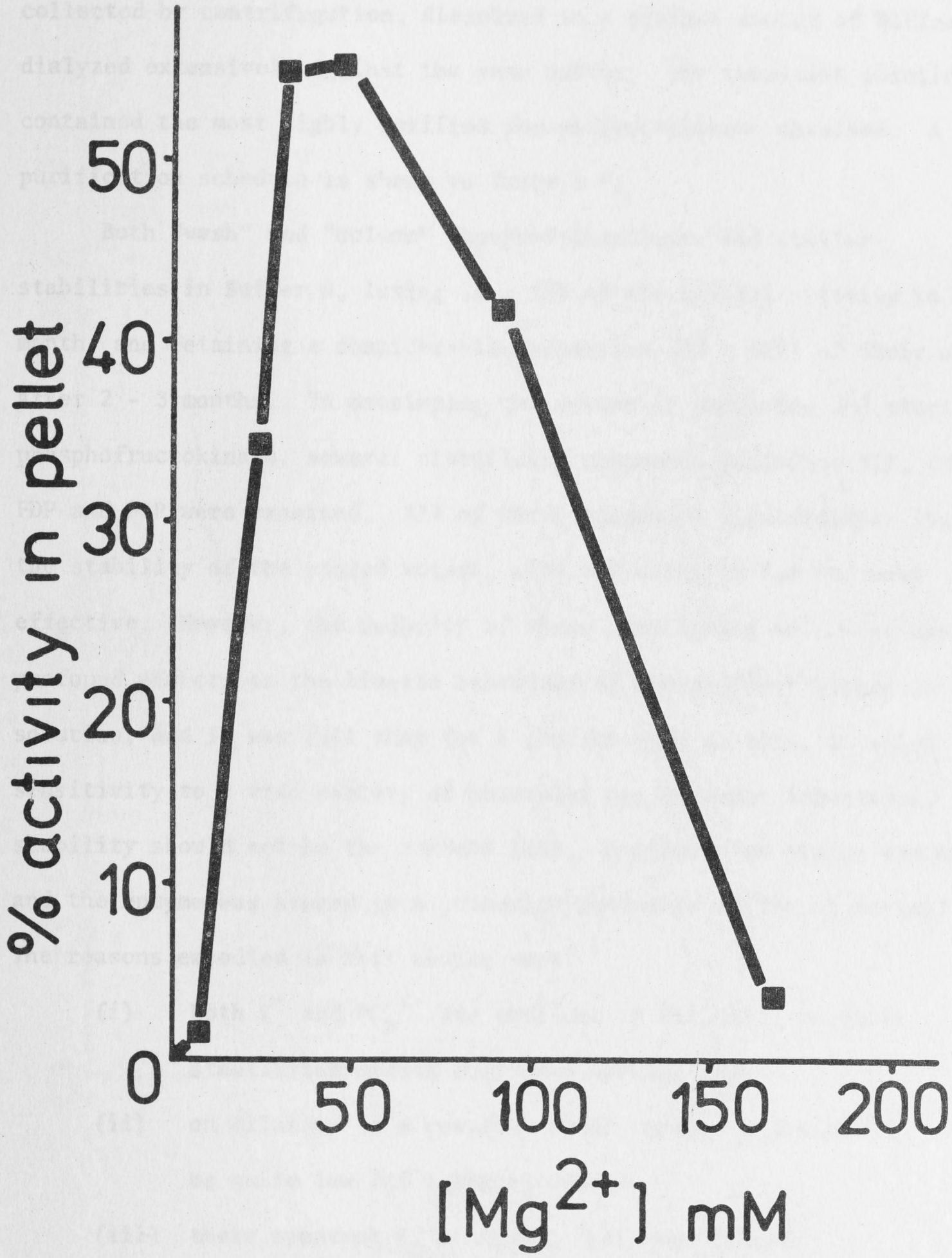
3. *Magnesium-precipitation* (see Figure 3-1). 1 M $MgCl_2$ (5 ml/100 ml solution) was slowly added to the supernatant, which was then stirred for 45 minutes in the cold. The enzyme-containing precipitate was collected by centrifugation for 15 minutes at 12,000 x g.

4. *Washing.* The red-brown pellets obtained were pooled and dispersed with a glass rod in 10 mls of Buffer B, then centrifuged for 15 minutes at 30,000 x g. The supernatant was poured off and the washing procedure repeated twice more. At this stage the enzyme-containing pellet was red-brown in colour, and the supernatant obtained after each washing had changed in colour from red to nearly colourless. Phosphofructokinase was solubilized from the pellet by addition of Buffer C, in the ratio of 1 ml/70 g starting material. After lengthy dispersal in this buffer, the solution was centrifuged and the supernatant decanted and stored. Prolonged dialysis against three changes of Buffer D, gave the "washed" enzyme, which was used for the majority of the kinetic experiments reported below. However, about 20% of the studies conducted were made with a more highly purified form of enzyme, prepared as described below.

5. *DEAE-cellulose column chromatography.* This step was introduced primarily to see if any different enzymatic forms of phosphofructokinase could be separated. The dialyzed "washed" enzyme was applied to a column (15 cm x 1.5 cm) containing DEAE-cellulose equilibrated with Buffer D, and was washed with 50 ml of the same buffer. A gradient was then introduced with the same buffer plus 0 - 500 mM $(NH_4)_2.SO_4$. Five ml samples were collected, and both the wash and gradient-elution fractions assayed for

Figure 3-1 The $Mg^{2+}$-precipitation boundaries of pig spleen phosphofructokinase

Phosphofructokinase was precipitated and solubilized as described in Section 3.4.1. Assays of maximal activity were conducted as in Section 3.3.1. The concentration used during the purification procedure is 50 mM.


% activity in pellet
0
10
20
30
40
50
50
100
150
200
[Mg2+] mM

phosphofructokinase activity and $NH_4^+$ content. Figure 3-2 shows the elution profile. Fractions containing most of the activity in each peak were pooled, and phosphofructokinase precipitated by adding solid $(NH_4)_2.SO_4$ to 65% saturation. After stirring overnight in the cold, precipitates were collected by centrifugation, dissolved in a minimal amount of Buffer D, and dialyzed extensively against the same buffer. The resultant solution contained the most highly purified phosphofructokinase obtained. A typical purification schedule is shown in Table 3-1.

Both "wash" and "column" phosphofructokinase had similar stabilities in Buffer D, losing 10 - 15% of the initial activity in one month, and retaining a considerable proportion (40 - 60%) of their activity after 2 - 3 months. In developing the system of preparing and storing phosphofructokinase, several stabilizing compounds including ATP, $(NH_4)_2.SO_4$, FDP and F6P were examined. All of these compounds significantly increased the stability of the stored enzyme, with FDP being by far the most effective. However, the majority of these stabilizing molecules have profound effects on the kinetic behaviour of phosphofructokinase in solution, and it was felt that for a project such as this, in which sensitivity to a wide variety of molecules was of major importance, stability should not be the supreme goal. A compromise choice was made, and the enzyme was stored in a potassium phosphate buffer at optimal pH. The reasons embodied in this choice were:

(i) both $K^+$ and $PO_4^{2-}$ are abundant in the cell, so their stabilizing effect will be a natural one;

(ii) on dilution in a cuvette, their concentration will be quite low (cf intracellular);

(iii) their apparent $K_D$'s suggest that they should instantly dissociate from the enzyme upon dilution; and

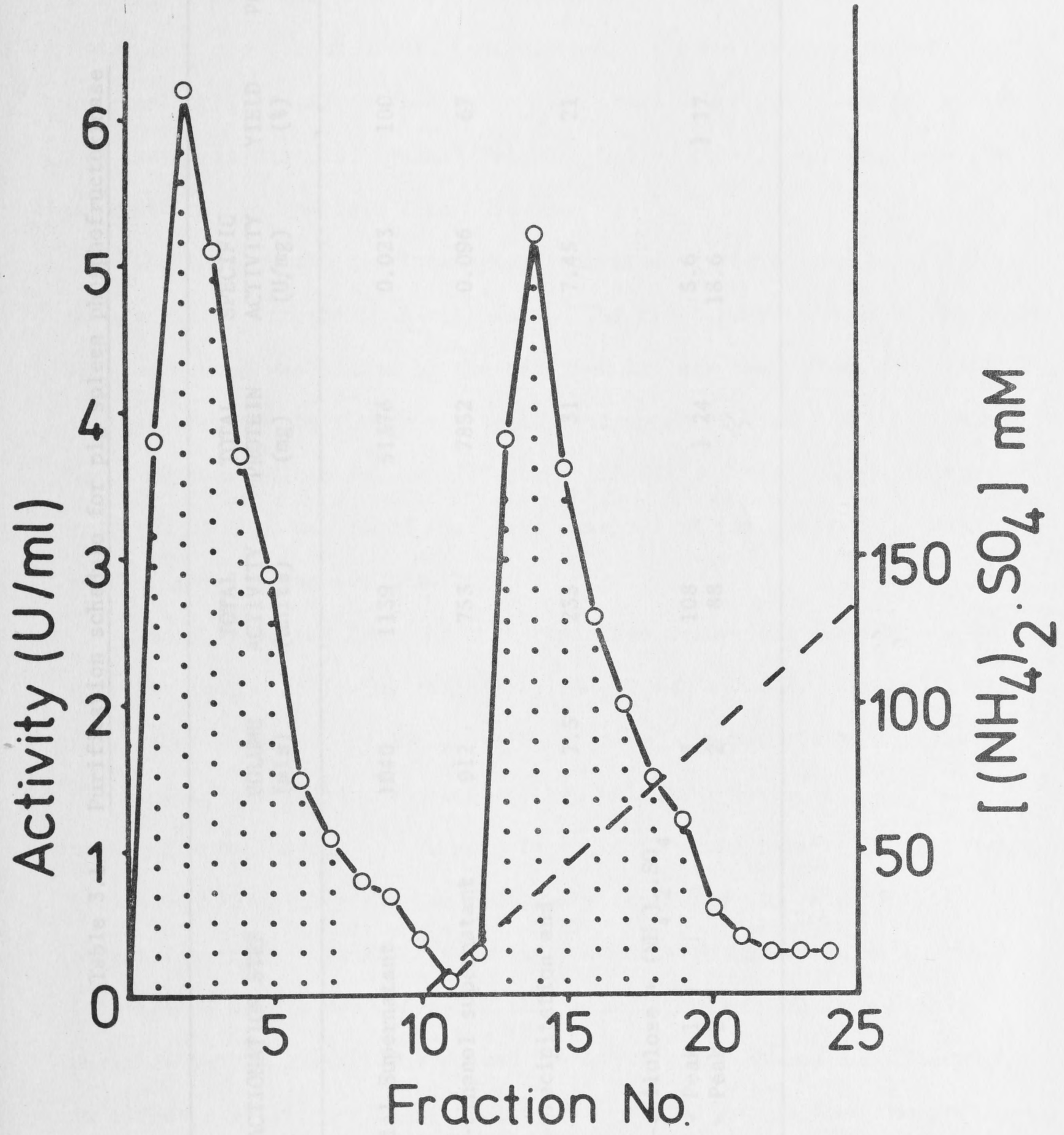



Figure 3-2 Elution of phosphofructokinase from DEAE-cellulose

Details are contained in the text. Fractions indicated with black dotted lines were pooled for $(NH_4)_2.SO_4$ precipitation.

Table 3.1 Purification schedule for pig spleen phosphofructokinase

| FRACTIONATION STEP | VOLUME (mls) | TOTAL ACTIVITY (units) | TOTAL PROTEIN (mg) | SPECIFIC ACTIVITY (U/mg) | YIELD (%) | PURIFICATION |
|---|---|---|---|---|---|---|
| 1. Initial Supernatant | 1040 | 1139 | 51376 | 0.023 | 100 | 1 |
| 2. Heat-ethanol supernatant | 912 | 753 | 7852 | 0.096 | 67 | 4 |
| 3. $Mg^{2+}$-precipitation and wash | 7.5 | 235 | 31 | 7.45 | 21 | 324 |
| 4. DEAE-cellulose + $(NH_4)_2.SO_4$ | | | | | | |
| - Peak 1 | 3 | 108 | } 24 | 5.6 | } 17 | 815 |
| - Peak 2 | 2 | 88 | | 18.6 | | (Peak 2) |

(iv) these stabilizers appeared to have less effect on the kinetic behaviour of phosphofructokinase than any of the other molecules tested.

Potassium phosphate will keep the enzyme active enough for only about 2 months, whereas the same buffer containing 0.2 mM ATP, 0.1 mM F6P, 2 mM $(NH_4)_2.SO_4$ and 0.2 mM dithiothreitol leaves the enzyme with 70% of its original activity after 4 months. Some stabilizers are essential as if the enzyme is dialyzed against Tris-Cl pH 8.0, it will rapidly lose its activity and is unusable after 3 days.

Neither of the two isoenzymes separated on the column displayed much affinity for the DEAE-cellulose. The first came through in the wash, and the second was eluted by the addition of less than 50 mM $(NH_4)_2.SO_4$. Initial attempts to purify rat spleen phosphofructokinase utilized the batch DEAE-cellulose method of Tarui *et al* (57). This procedure was rejected when it was found that less than 50% of the activity in the initial supernatant was adsorbed.

Each isoenzyme from the DEAE-cellulose column was compared in its response to the following effectors: $NH_4^+$, AMP and ATP. Kemp (54) has shown that responses to AMP and ATP addition, demonstrate most clearly the kinetic differences between the two principal forms of phosphofructokinase, — "muscle-type", and "liver-type". On this basis it was concluded that the two isoenzymes separated were kinetically almost identical, and all future experiments were conducted with either a 1:1 mixture of the two types, or the "wash" enzyme. The more highly purified enzyme prepared by column chromatography, showed no differences in either stability or kinetic properties to the enzyme from the previous "wash" stage.

### 3.4.2 Justification of the assay method

The assay system, in most of the experiments reported in Chapter 3,

has depended upon the quantitative conversion of the product of the phosphofructokinase reaction, FDP, via 3 coupling enzymes, to α-glycerophosphate, with the subsequent oxidation of NADH. Although such coupled enzyme assays are described routinely in the literature, it was felt advisable to check the validity of the method in the present system.

Figure 3-3 compares the reaction rates determined with the coupled enzyme method, and the direct assay method. Cuvette contents were as described in Section 3.3.1 for examination of regulatory properties. For direct assay, reaction mixtures were pipetted into cuvettes which contained no coupling enzymes. Reactions were initiated by the addition of phosphofructokinase, and at 15 second intervals were stopped by the addition of 100 µL 5N HCl (lowers pH to <2.0). Samples were mixed thoroughly and stood for 30 seconds before neutralization with 110 µL 5N KOH. The optical density was read and the total amount of FDP formed was determined by addition of coupling enzymes, and measuring the oxidation of NADH. Corrections were made for the slight volume differences. Clearly, under the conditions used, the two methods gave identical results.

### 3.4.3 Some general properties of phosphofructokinase

In this section some of the more general properties of phosphofructokinase are presented. Figure 3-4 shows the sigmoidal response of phosphofructokinase to increasing F6P concentration, when assayed at pH 7.0 in the presence of fixed concentrations of MgATP. The sigmoidicity observed is a function of the MgATP concentration. When 0.8 mM MgATP was present, the Hill Coefficient was calculated to be 1.41, and when 1.5 mM MgATP was used, the Hill Coefficient increased to 1.79. As presented in the diagram, it would appear that the maximal velocity observed is also a function of the MgATP concentration. However, such an interpretation is superficial, as, when the protein concentration is doubled, maximal velocities at both concentrations of MgATP are increased,

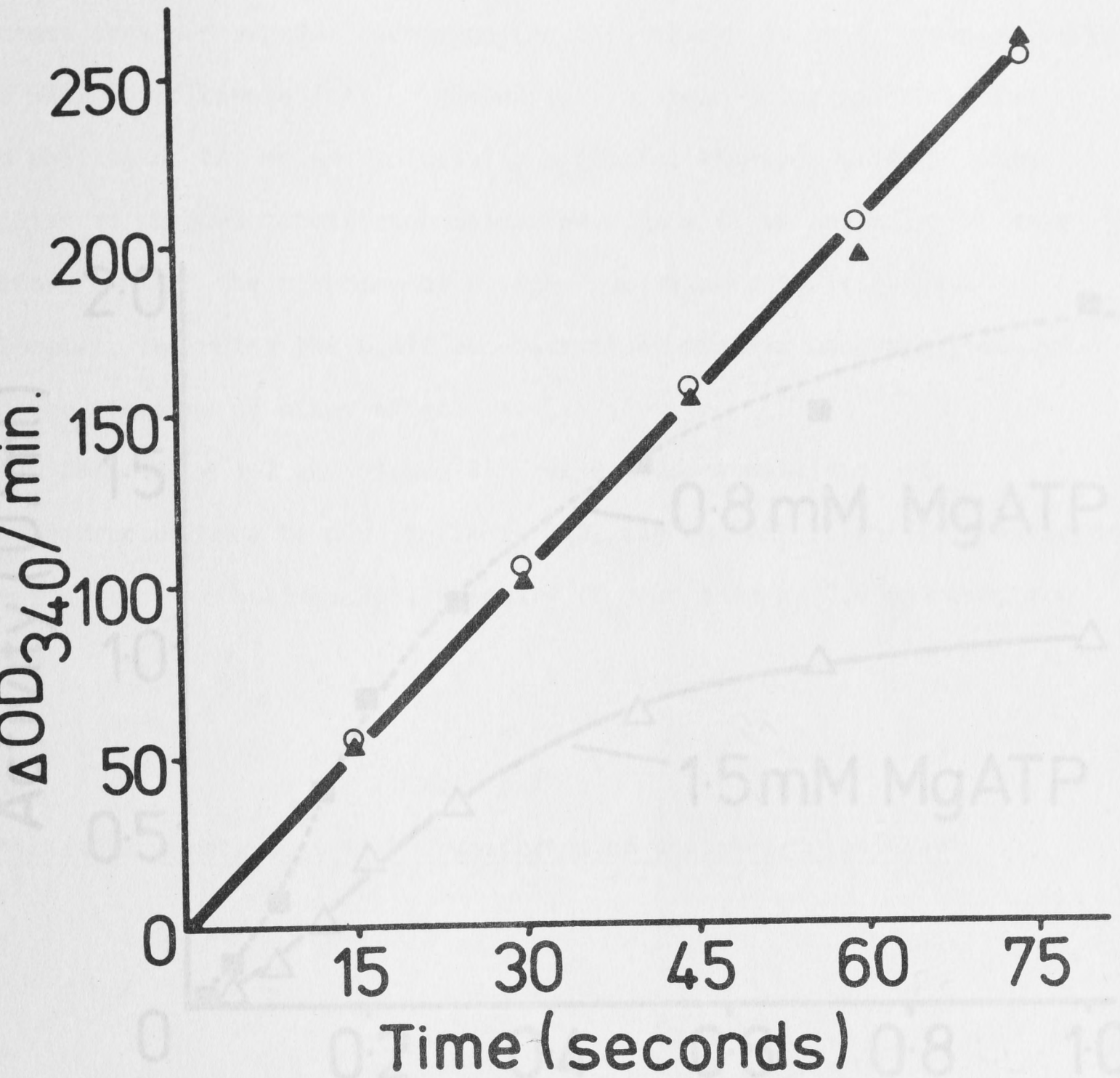



Figure 3-3 Comparison of coupled and direct enzyme assays

Conditions as described in the text.

o coupled, ▲ direct.

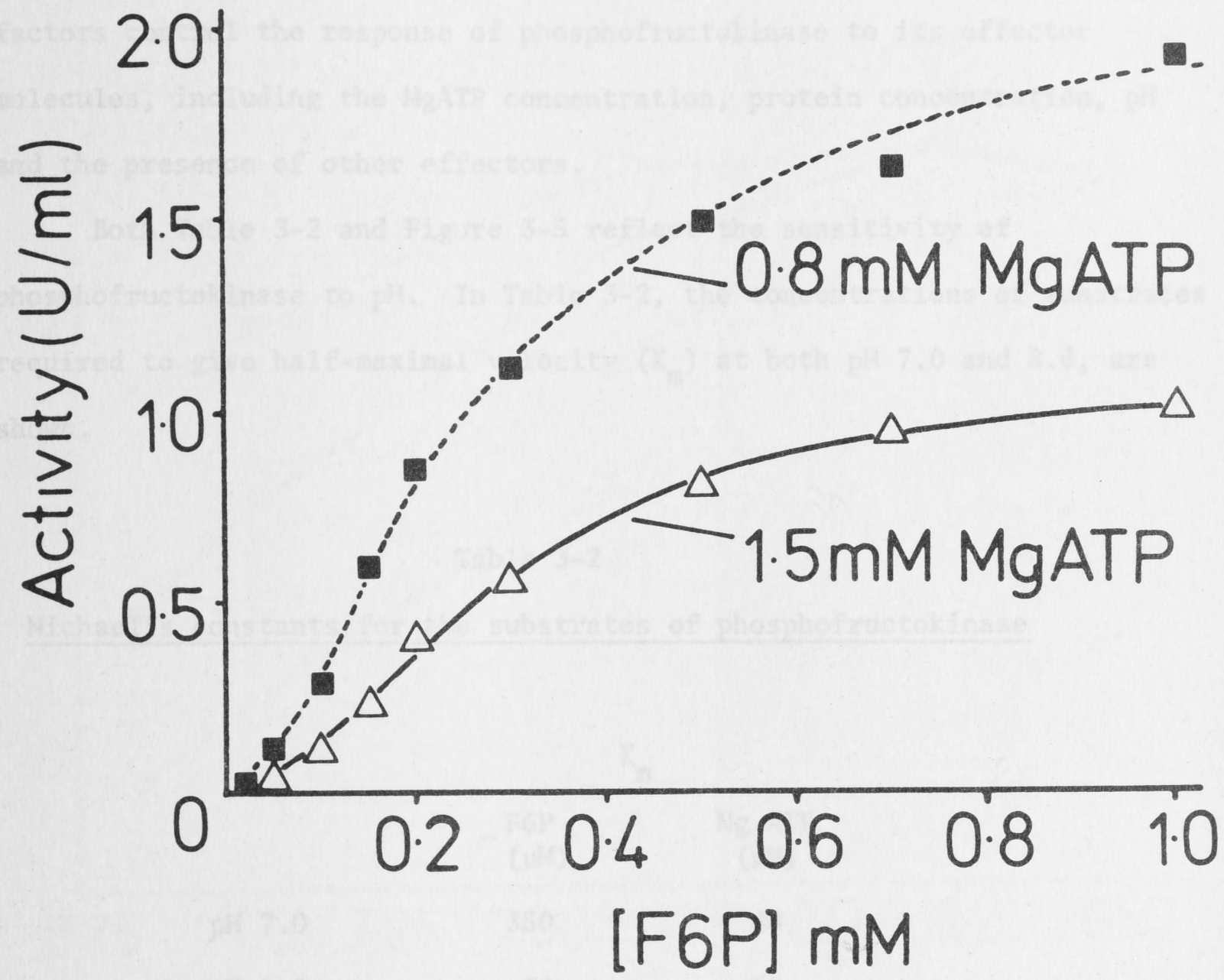



Figure 3-4 The sigmoidal response of phosphofructokinase to F6P

Conditions of assay as described in the text.

and the disparity between the velocities recorded at high F6P concentration becomes less pronounced. Accompanying this change in protein concentration, the Hill Coefficients fall. This effect is shown later in Table 3-14. The ability of the enzyme to display sigmoidal kinetics parallels its ability to respond to effector molecules. As will be shown later, many factors control the response of phosphofructokinase to its effector molecules, including the MgATP concentration, protein concentration, pH and the presence of other effectors.

Both Table 3-2 and Figure 3-5 reflect the sensitivity of phosphofructokinase to pH. In Table 3-2, the concentrations of substrates required to give half-maximal velocity ($K_m$) at both pH 7.0 and 8.0, are shown.

Table 3-2

Michaelis constants for the substrates of phosphofructokinase

| | $K_m$ | |
|---|---|---|
| | F6P (μM) | Mg.ATP (μM) |
| pH 7.0 | 350 | 40 |
| pH 8.0 | 32 | 60 |

At each pH, one substrate was held steady at constant, saturating levels, whilst the activity was titrated with respect to the second substrate. When the $K_m$ for F6P was measured, the MgATP concentration was held steady at 2.0 mM, and when the $K_m$ for MgATP was measured, the F6P level was fixed at 2.0 mM. Of greatest interest is the large increase in $K_m$ for F6P as the pH decreases from 8.0 to 7.0. When the normal *in vivo* levels of F6P are considered (see Table 2-3), it is apparent that in the presence of

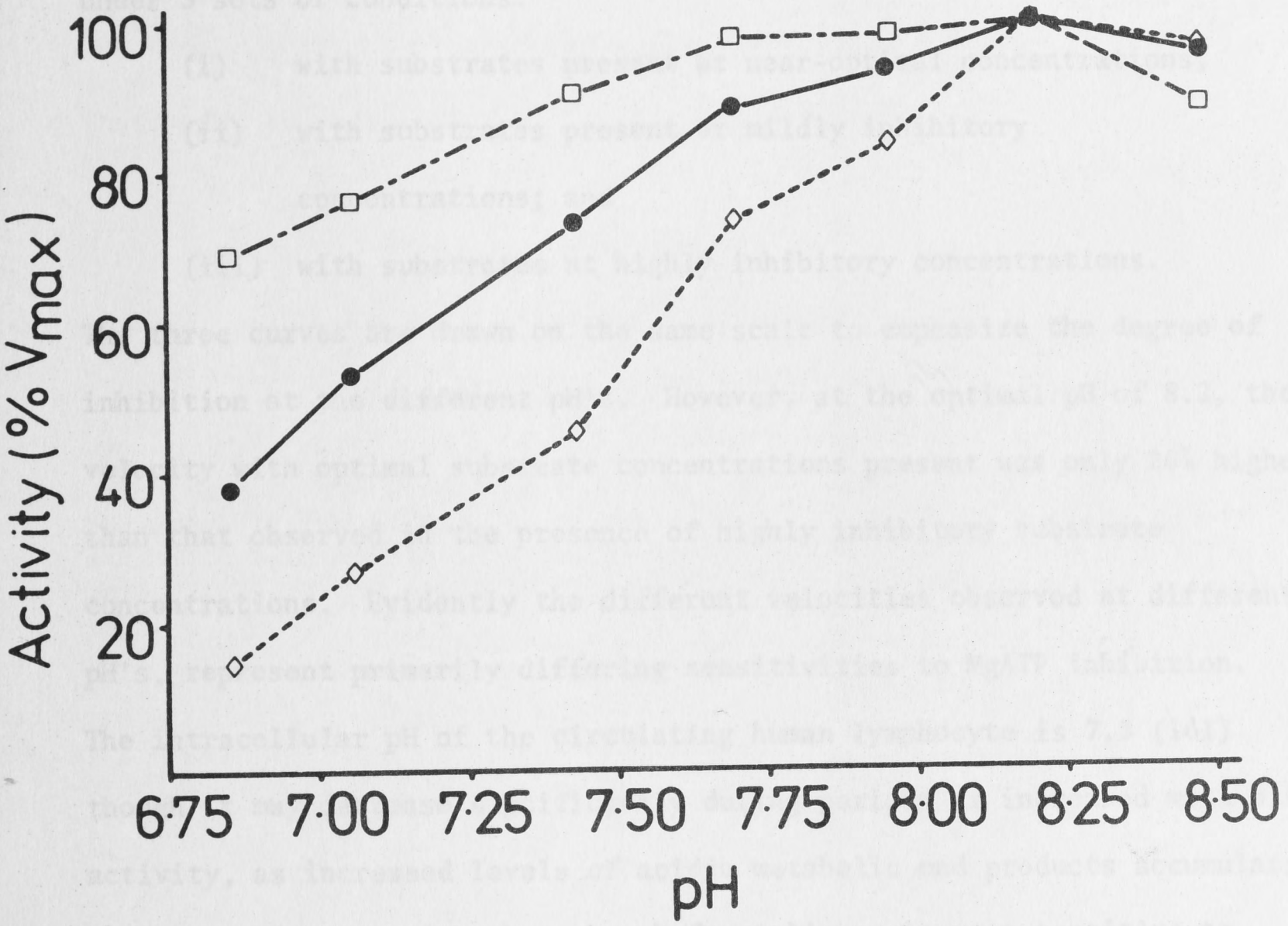



Figure 3-5 The effect of pH on phosphofructokinase activity

Assays were conducted at the indicated pH, as described in Section 3.3.1.

----◇--- 0.2 mM F6P, 2.0 mM Mg.ATP;

——●—— 0.2 mM F6P, 0.4 mM Mg.ATP;

—·—□—· 1.0 mM F6P, 0.2 mM Mg.ATP.

physiological concentrations of ATP, phosphofructokinase will be very inhibited. Although no values are shown here, the $K_m$ for F6P at pH 7.0 increases as the MgATP concentration increases. Kemp (54), has also observed this. In his system, increasing the MgATP concentration from 0.5 to 2.0 mM raised the $K_m$ for F6P 2-3 fold.

In Figure 3-5, phosphofructokinase was assayed at different pH values under 3 sets of conditions:

(i) with substrates present at near-optimal concentrations;

(ii) with substrates present at mildly inhibitory concentrations; and

(iii) with substrates at highly inhibitory concentrations.

The three curves are drawn on the same scale to emphasize the degree of inhibition at the different pH's. However, at the optimal pH of 8.2, the velocity with optimal substrate concentrations present was only 20% higher than that observed in the presence of highly inhibitory substrate concentrations. Evidently the different velocities observed at different pH's, represent primarily differing sensitivities to MgATP inhibition. The intracellular pH of the circulating human lymphocyte is 7.3 (161) though it may decrease significantly during periods of increased metabolic activity, as increased levels of acidic metabolic end products accumulate. It is in this pH region that phosphofructokinase is most sensitive to inhibition by MgATP.

### 3.4.4 Survey of effector molecules

In this section, a variety of potential effector molecules have been tested. This is not intended to be an exhaustive examination of all possible effectors, but is an attempt to scan several *families* of molecules, members of which have been reported previously to be effectors of phosphofructokinase, or for which some regulatory role might be anticipated on teleological grounds. For this survey, all assays were conducted at

pH 7.0, a pH at which the regulatory properties of the enzyme are most easily visualized. Concentrations used are not intended to approximate physiological levels in any way, but are high enough to ensure that any potential effect will be seen. Any molecules which were found to significantly alter phosphofructokinase activity were subsequently examined in greater detail, and these results recorded in Sections 3.4.5 and 3.4.6 below.

The ability of the glycolytic intermediates to alter phosphofructokinase activity is shown in Table 3-3.

Table 3-3

The effect of glycolytic intermediates on phosphofructokinase activity

Assays were conducted at pH 7.0 with 0.2 mM F6P and 1.0 mM MgATP. For assay of FDP effect, an excess of dialyzed pyruvate kinase, and lactate dehydrogenase in glycerol suspension was added, with 0.8 mM PEP. Activity was observed as the oxidation of NADH at 340 n.m.

| ADDITION | | RELATIVE ACTIVITY |
|---|---|---|
| None | | 100 |
| D-Glucose | (4 mM) | 107 |
| G6P | (1.3 mM) | 91 |
| FDP | (0.3 mM) | 122 |
| 3-PGA | (2.0 mM) | 62 |
| 2-PGA | (2.3 mM) | 78 |
| PEP | (2.0 mM) | 40 |
| Pyruvate | (2.5 mM) | 105 |

All intermediates except F6P, the triose-phosphates and 1,3-diPGA were

tested. The ability of F6P to activate phosphofructokinase was discussed earlier in Chapter 1 of this thesis. More will be said of its role later. Of the intermediates examined, glucose, G6P and pyruvate appeared to be ineffective, whilst FDP was a weak activator, and 3-PGA, 2-PGA and PEP were inhibitory to varying degrees. FDP is reported to be a potent activator of phosphofructokinase from several mammalian tissues (52, 58). At no time, and under no conditions during the present study, did it increase activity by more than 40%. These results were reproducible, and it was concluded that the low degree of activation was a real property of the spleen enzyme. The inhibition by the phosphoglycerates and PEP has been observed for phosphofructokinases isolated from several tissues (49, 59). The concentrations of these molecules were far higher than is found in lymphoid tissues *in vivo*. A more detailed study of the kinetics of this inhibition is presented in Section 3.4.6 below.

Suter (20), during his investigations into the fuels of respiration of the rat spleen slice, found that the levels of some endogenous amino acids changed when incubations were conducted in the presence as opposed to the absence, of glucose. The only one which changed significantly was aspartate, the levels of which fell from 13.2 to 9.5 μ moles/g. dry wt., when slices were incubated for 60 minutes with 3 mM glucose. The levels of several other amino acids also changed, but with such large standard errors that the differences were not significant. Table 3-4 shows how these amino acids alter phosphofructokinase activity. Clearly none are inhibitory, and glutamine and aspartate appear to be stimulatory. So far as is known, this is the first report of an amino acid being an effector for phosphofructokinase. A study of the kinetics of activation by aspartate is presented in Section 3.4.5.

Table 3-4

Effect of some amino acids on phosphofructokinase activity

Assay conditions as for Table 3-3.

| ADDITION | | RELATIVE ACTIVITY |
|---|---|---|
| None | | 100 |
| Glutamate | (2.5 mM) | 102 |
| Glutamine | (2.5 mM) | 135 |
| Glycine | (2.5 mM) | 112 |
| Aspartate | (2.5 mM) | 160 |
| Alanine | (2.5 mM) | 113 |

Regulation of phosphofructokinase activity by adenine nucleotides has been well documented. As shown in Table 3-5, ATP is the only nucleotide to significantly inhibit phosphofructokinase, whilst ADP, AMP and cAMP are the only nucleotides to cause a large activation.

Table 3-5

Effect of some nucleotides and nucleosides on phosphofructokinase

Assay conditions as for Table 3-3.

| ADDITION | | RELATIVE ACTIVITY |
|---|---|---|
| None | | 100 |
| ATP | (2.0 mM) | 55 |
| ADP | (1.0 mM) | 125 |
| AMP | (1.0 mM) | 195 |
| cAMP | (1.0 mM) | 161 |
| Adenosine | (2.5 mM) | 116 |
| IDP | (2.5 mM) | 89 |
| IMP | (2.5 mM) | 87 |
| Inosine | (2.5 mM) | 103 |
| cGMP | (0.2 mM) | 102 |

Other nucleoside triphosphates (NTP's) were examined, (Table 3-6), but clearly it is only the *adenine* nucleotides which have significant effects on enzyme activity. A detailed study of their effects is presented in Sections 3.4.5 and 3.4.6.

A related study, — that of the ability of other NTP's to act as substrates and inhibitors of phosphofructokinase, is presented in Table 3-6.

Table 3-6

The ability of various nucleoside triphosphates to act as substrates and inhibitors of phosphofructokinase

Assays conducted at pH 7.0, as described in Section 3.3.1, with 0.2 mM F6P and $Mg^{2+}$ 3 mM in excess of the NTP.

| NUCLEOTIDE | ACTIVITY+ IN 0.3 mM NTP | ACTIVITY+ IN 3.0 mM NTP | $\frac{V_L}{V_H}$ |
|---|---|---|---|
| ATP | 100* | 22 | 4.55 |
| dATP | 15 | <1 | — |
| ITP | 89 | 83 | 1.07 |
| GTP | 103 | 67 | 1.54 |
| UTP | 54 | 42 | 1.29 |
| CTP | 47 | 34 | 1.38 |

+All values are expressed as a percentage of the activity in 0.3 mM ATP.

*0.3 mM ATP is already mildly inhibitory.

It must be understood that in the following section, when a NTP is referred to as being a substrate or inhibitor, it is acting in this manner only when bound as a $Mg^{2+}$-nucleotide complex. Several workers have

considered this question, with varying results. Uyeda and Racker (49) found that ATP, ITP, UTP and CTP would all act as $PO_4^{2-}$ donors for the reaction catalyzed by skeletal muscle phosphofructokinase and, furthermore, that both ATP and UTP were highly inhibitory at high concentrations. Neither ITP nor CTP showed much inhibitory capacity. Lowry and Passoneau (58) found that the brain enzyme could use all the above NTP's as $PO_4^{2-}$ donors, and GTP and TTP as well. Of all these, ATP, ITP and UTP were inhibitory. In Table 3-6, all the NTP's were tested at a low concentration where they should nearly maximally activate the enzyme, and also at a concentration ten times higher, to see if any inhibitory effect was apparent. ATP, GTP and ITP appeared to be good substrates, UTP and CTP decidedly less efficient ones, and dATP a very poor one. Both ATP and dATP were strongly inhibitory at the higher concentration, whilst GTP, CTP and UTP were only slightly inhibitory. ITP apparently serves only as a substrate. Both Uyeda and Racker (49), and Lowry and Passoneau (58) found UTP to be inhibitory, but disagreed as to the effectiveness of ITP as an inhibitor. The results presented here agree with Uyeda and Racker that ITP is non-inhibitory, but are in contradiction to both groups in finding that UTP is only weakly inhibitory.

Table 3-7 contains a miscellany of effector molecules: four Krebs-cycle intermediates, P-creatine (which is reported to be an inhibitor of phosphofructokinase) and two radicals $PO_4^{2-}$ and $SO_4^{2-}$ which are also thought to be effectors for the enzyme. Citrate and succinate inhibit phosphofructokinase to a considerable extent, though at high concentrations. Malate is a poor inhibitor, and fumarate ineffective at the same concentrations. In all reports published to date, citrate, of all the Krebs'-cycle intermediates has been shown to be the most effective inhibitor of phosphofructokinase.

Table 3-7

Effect of some Krebs' cycle intermediates and other metabolites on phosphofructokinase activity

Assay conditions as for Table 3-3.

| ADDITION | | RELATIVE ACTIVITY |
|---|---|---|
| None | | 100 |
| Citrate | (2.0 mM) | 40 |
| Fumarate | (2.5 mM) | 90 |
| Malate | (2.5 mM) | 76 |
| Succinate | (2.5 mM) | 65 |
| $SO_4^{2-}$ | (3.0 mM) | 112 |
| $PO_4^{2-}$ | (3.0 mM) | 109 |
| P-creatine | (1.5 mM) | 112 |

As noted in Chapter 1, as far as inhibitors are concerned, it is very difficult to make quantitative comparisons with the results of other workers. However, Kemp (54) in his comparison of liver and muscle phosphofructokinases, found that the muscle enzyme was extremely sensitive to citrate, being almost totally inhibited by 0.5 mM, whilst the liver enzyme was less than 50% inhibited by 2 mM citrate. It is reasonable to suggest from the data in Table 3-7, that spleen phosphofructokinase is more similar in this respect to the liver than the muscle enzyme. A more detailed study of the citrate inhibition is presented in Section 3.4.6. Passoneau and Lowry (92) and Underwood and Newsholme (60) have also looked at the effects of Krebs'-cycle intermediates on phosphofructokinase activity. In agreement with the results presented above, they also found citrate to be the most effective inhibitor, whilst malate and succinate

were weakly inhibitory, and fumarate ineffective.

P-creatine is reported to inhibit phosphofructokinase activity (49). However, from Table 3-7 it is apparent that no inhibition can be observed. Kemp (54) also found P-creatine to have little effect on enzyme activity. $PO_4^{2-}$ and $SO_4^{2-}$ have only a small stimulatory effect on phosphofructokinase, but an additional property of both radicals, namely an ability to protect the enzyme from inactivation by MgATP, has led to a more detailed study of their effect. These results are contained in Section 3.4.7.

MgATP is the natural substrate for phosphofructokinase. Muntz (45) reported that the brain enzyme could also use MnATP and CoATP as substrates. In Table 3-8 the ability of several divalent metal ions to substitute for $Mg^{2+}$ is tested.

Table 3-8

The ability of some divalent metal ions to replace $Mg^{2+}$ as substrate for the phosphofructokinase reaction

Assays were conducted as described in Table 3.3, save that instead of an excess of $Mg^{2+}$, the particular metal ion, at the indicated concentration, was added.

| ADDITION | | RELATIVE ACTIVITY |
|---|---|---|
| $MgCl_2$ | (1.5 mM) | 100 |
| $CaCl_2$ | (1.5 mM) | 0 |
| $MnCl_2$ | (1.5 mM) | 63 |
| $CoCl_2$ | (1.5 mM) | 39 |
| $SrCl_2$ | (1.5 mM) | 1 |
| $CdCl_2$ | (1.5 mM) | 0 |
| $BaCl_2$ | (1.5 mM) | 0 |

Instead of the normal 3 mM excess of metal ion over ATP, only 1.5 mM metal ion was added in the presence of 1.0 mM ATP. This was done to ensure that any inhibition by free metal ions was kept to a minimum. As with the brain enzyme, $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ (as the Me-ATP complexes) were able to act as substrates for phosphofructokinase. Figure 3-6 shows the effect on enzyme activity, when these three metal ions are titrated at a fixed concentration of ATP. To clearly understand the significance of this data, it is necessary to refer to Table 3-12 in Section 3.4.7 later in this thesis. This shows that both free $Mg^{2+}$ and free ATP inhibit phosphofructokinase, but that free ATP is more inhibitory than free $Mg^{2+}$. As referred to in Section 3.3.1, an excess of divalent metal ion is required before all of the ATP is quantitatively bound. Referring back to Figure 3.6, it can be seen that, as $Mg^{2+}$ exceeds the total ATP concentration, activity continues to rise. Under these circumstances free $Mg^{2+}$ will be increasing rapidly, whilst free ATP levels will be low and falling. The conclusion to be drawn here is that free ATP is so highly inhibitory, that removing it, even by adding a large excess of another *inhibitor* (free $Mg^{2+}$) causes an increase in activity. This data could be explained in another way, namely that there are two allosteric sites on the enzyme for free $Mg^{2+}$, one an activator site with a high affinity for $Mg^{2+}$, the other an inhibitor site, with a low affinity. However, considering the data in Table 3-12, and the results of Lowry and Passoneau (58), the above conclusion seems more appropriate. When, however, either $Mn^{2+}$ or $Co^{2+}$ are titrated at a single concentration of ATP, maximal activity is reached when ATP and either $Mn^{2+}$ or $Co^{2+}$ are present in a ratio of approximately 1:1. This suggests that $Mn^{2+}$ and $Co^{2+}$ are inhibitory to the same degree as ATP, and that all three species are far more inhibitory than $Mg^{2+}$.

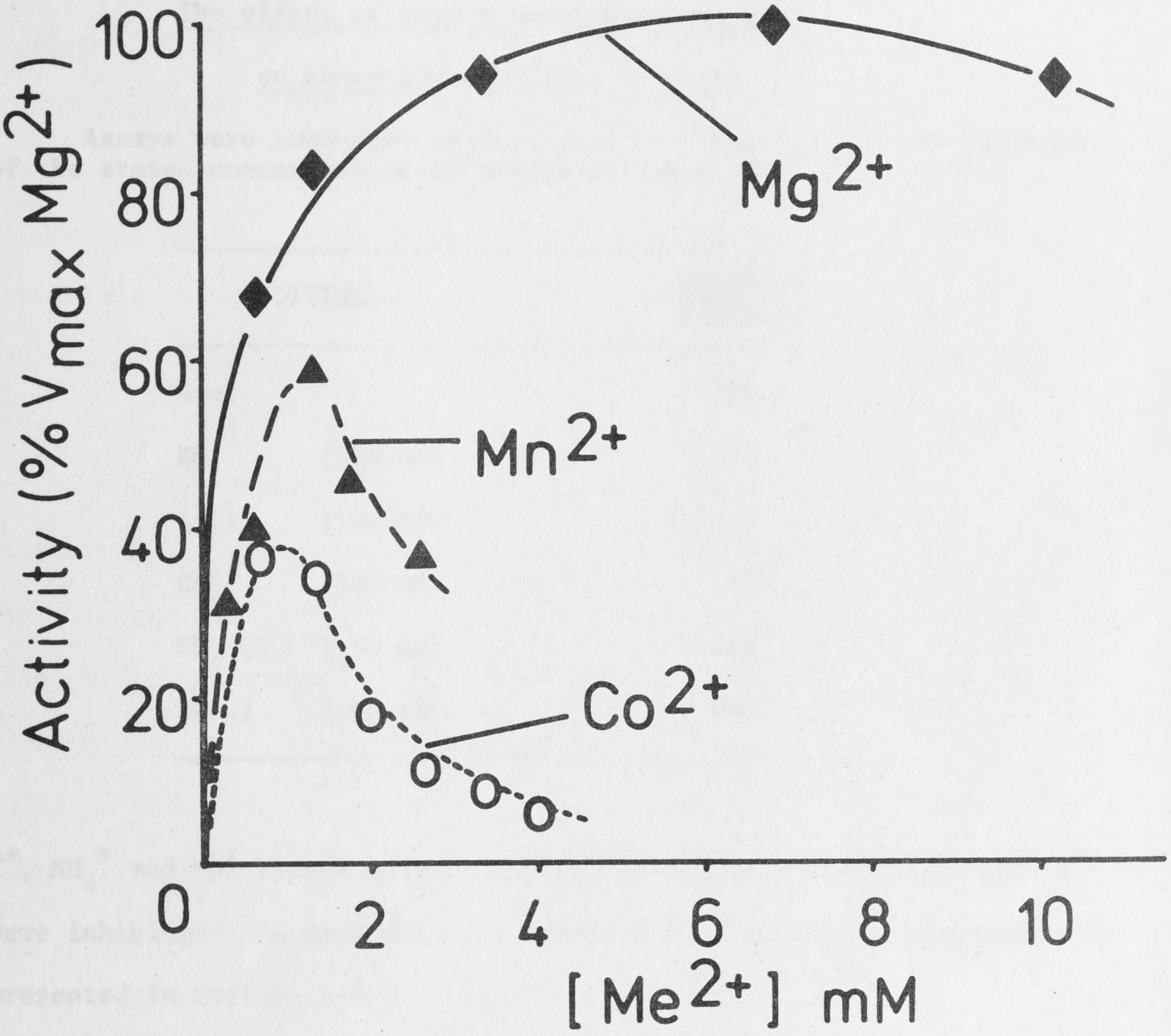



Figure 3-6 The ability of $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ to participate in the phosphofructokinase reaction

Assays were conducted as in Table 3-3, save that the metal ion concentrations were varied as indicated.

Both $K^+$ and $NH_4^+$ are potent stimulators of phosphofructokinase activity (83). Table 3-9 lists several other monovalent cations which were tested for their effect on the enzyme.

Table 3-9

The effect of some monovalent metal ions on phosphofructokinase activity

Assays were conducted as described in Table 3-3, in the presence of the stated concentration of monovalent metal ion.

| ADDITION | | RELATIVE ACTIVITY |
|---|---|---|
| None | | 100 |
| KCl | (100 mM) | 870 |
| LiCl | (100 mM) | 11 |
| CsCl | (100 mM) | 30 |
| $NH_4$.Cl | ( 10 mM) | 680 |
| Rb.Cl | (100 mM) | 430 |

$K^+$, $NH_4^+$ and $Rb^+$ showed strong stimulatory effects, whilst $Li^+$ and $Cs^+$ were inhibitory. A more detailed study of the kinetics of activation is presented in Section 3.4.5.

### 3.4.5 Activators

In Section 3.4.4, aspartate, AMP, cAMP, $K^+$, $NH_4^+$ and $Rb^+$ were all shown to markedly stimulate phosphofructokinase activity. In addition, FDP, glutamine, ADP and possibly $PO_4^{2-}$, $SO_4^{2-}$ and P-creatine gave some signs of activator potential. In this section, a more detailed examination will be made of the kinetics of activation by the more important of these effectors.

In Figure 3-7, the ability of $K^+$, $NH_4^+$ and $Rb^+$ to stimulate phosphofructokinase activity is demonstrated. In order of stimulatory capacity, $K^+ > NH_4^+ > Rb^+$. Of greatest interest is the difference in apparent activation constants ($K_A$'s) for the three ions. Both $K^+$ and $Rb^+$ have $K_A$'s of the order of 10 - 12 mM, whilst for $NH_4^+$ this value is only 0.35 mM. There is some suggestion that a high concentration of each cation causes a slight inhibition of enzyme activity.

Regulation of phosphofructokinase activity by adenine nucleotides is well documented (162, 163). Perhaps the single most important factor governing phosphofructokinase activity *in vivo*, is its almost total inhibition in the presence of the very high levels of ATP and very low levels of F6P found in living tissue. Newsholme (163) has pointed out that regulation of phosphofructokinase activity by variations in ATP levels alone would be unrealistic, as the extent of the necessary change would affect too many other energy-requiring processes within the cell. However, small percentage changes in ATP levels are amplified via the adenylate kinase equilibrium into much larger percentage changes in the levels of ADP and AMP, both activator molecules. Figure 3-8 shows the ability of ADP, AMP and cAMP to activate phosphofructokinase. The calculated $K_A$'s for these three nucleotides were 0.035 mM, 0.028 mM and 0.031 mM respectively. In order of stimulatory capacity; AMP > cAMP > ADP.

Figure 3-9 shows the activation of phosphofructokinase by aspartate. The apparent $K_A$ was calculated to be 0.7 mM. So far as is known, there have been no previous reports of activation by aspartate.

Both FDP and $PO_4^{2-}$ have been reported to significantly activate phosphofructokinase (58). However, as was mentioned briefly in Section 3.4.4, they were not observed to have any great stimulatory effect on the pig spleen enzyme. What was noted with $PO_4^{2-}$ and indirectly with FDP, was that these regulators appear to be most effective in blocking inhibition

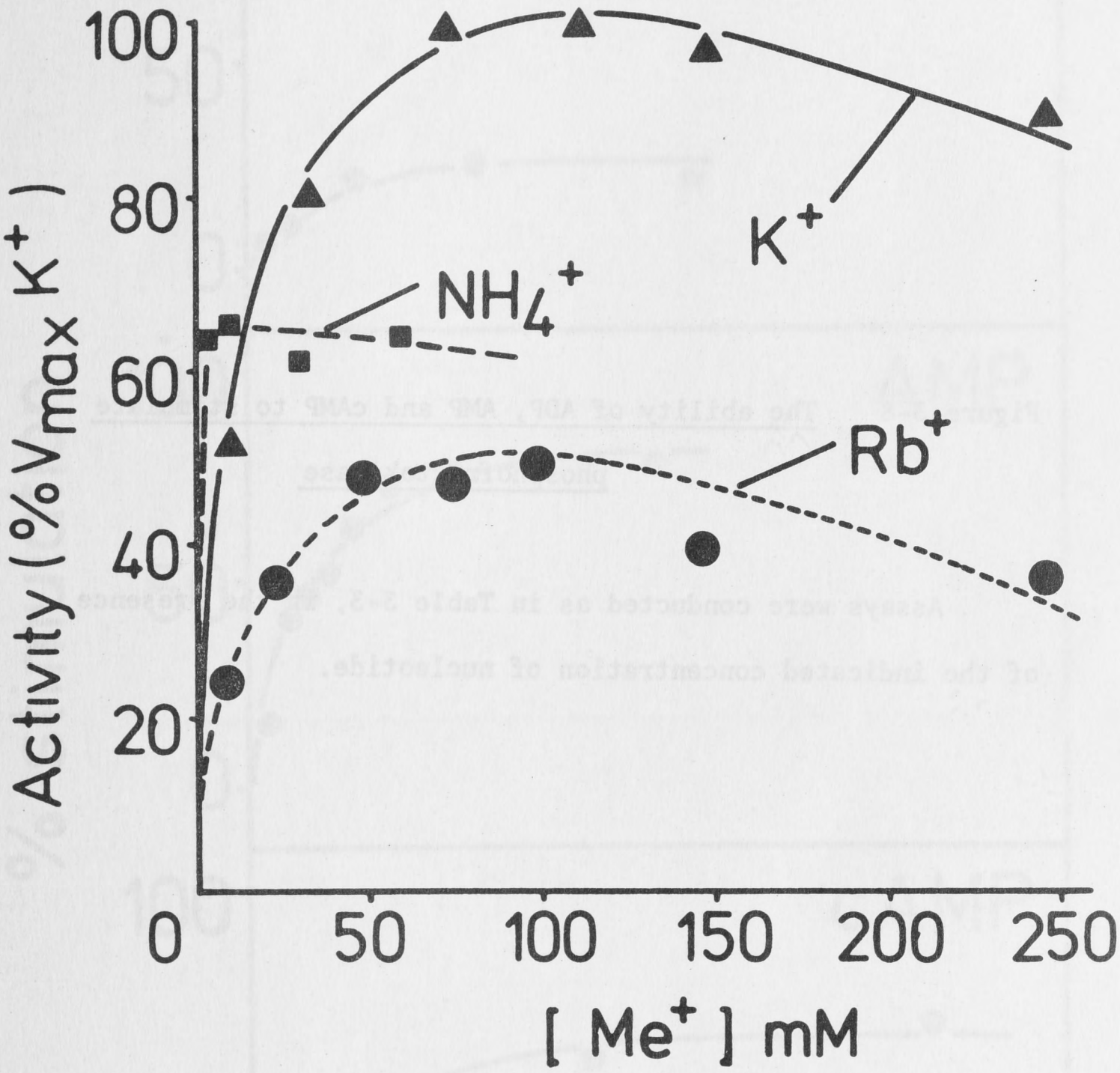
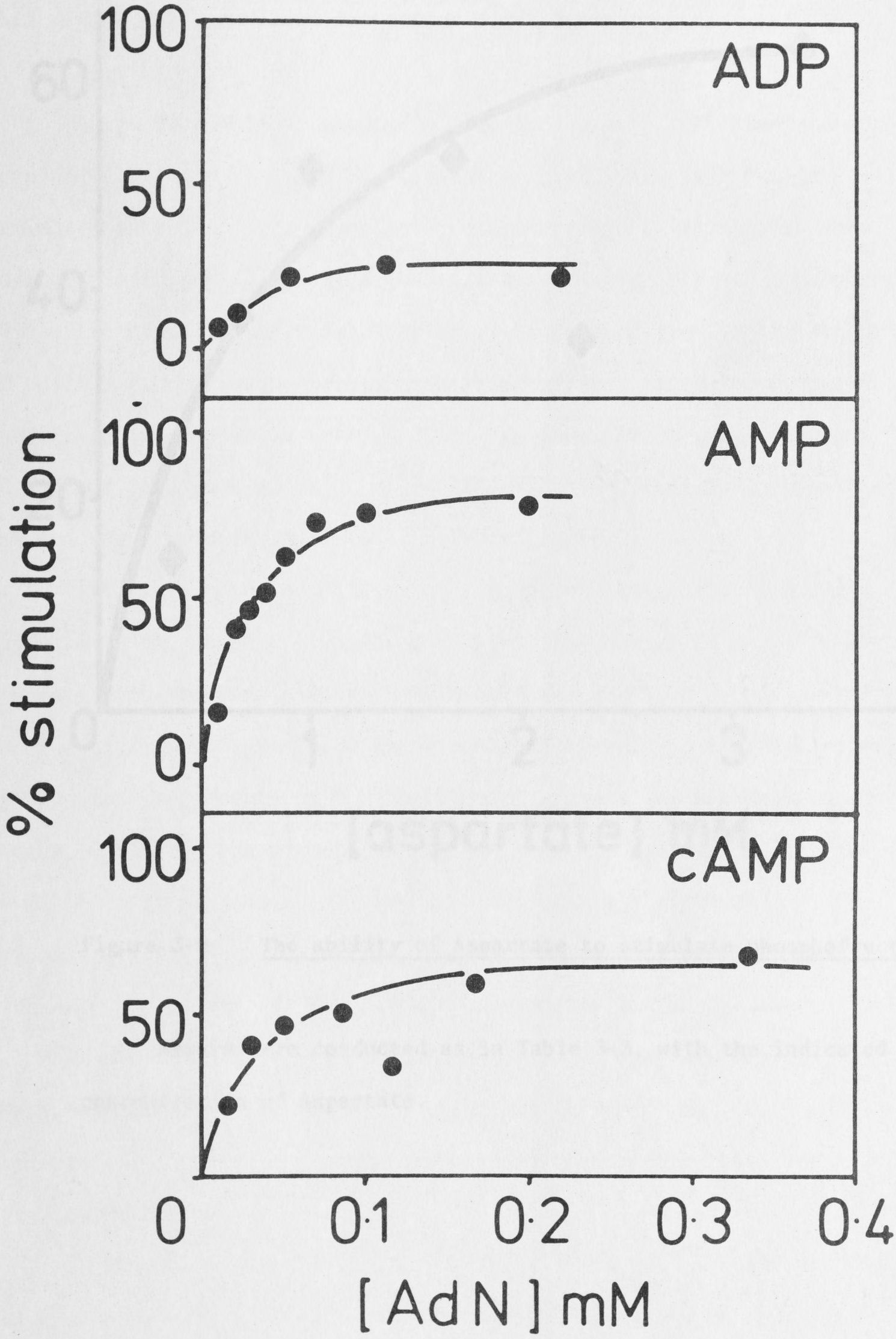



Figure 3-7 The ability of $K^+$, $NH_4^+$ and $Rb^+$ to stimulate phosphofructokinase

Assays were conducted as described in Table 3-3, save that the monovalent metal ion concentrations were varied as indicated.

Figure 3-8 The ability of ADP, AMP and cAMP to stimulate phosphofructokinase

Assays were conducted as in Table 3-3, in the presence of the indicated concentration of nucleotide.


ADP
AMP
cAMP
100
50
0
% stimulation
0
0·1
0·2
0·3
0·4
[AdN] mM

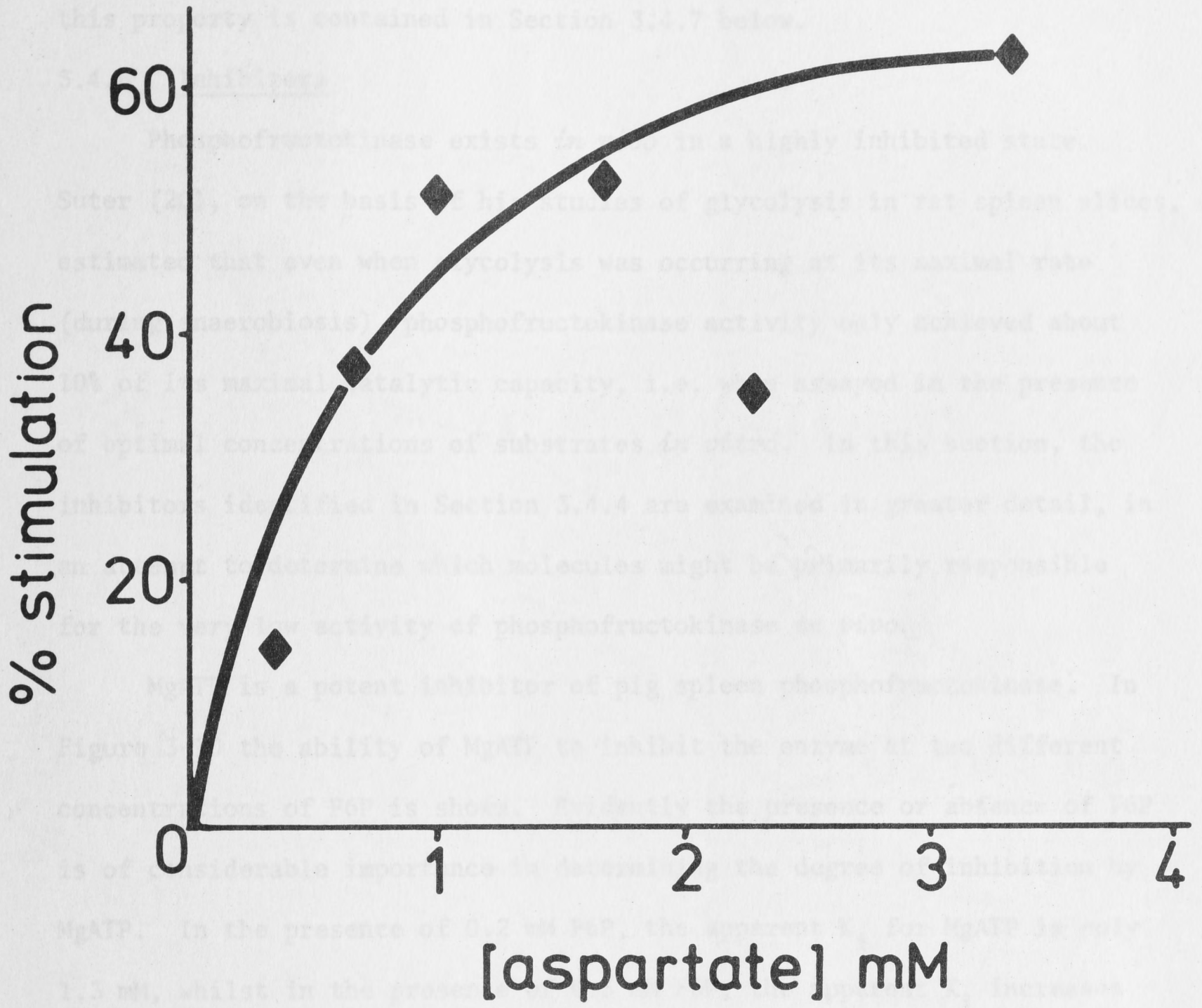



Figure 3-9 The ability of Aspartate to stimulate phosphofructokinase

Assays were conducted as in Table 3-3, with the indicated concentration of Aspartate.

or inactivation by MgATP. On the basis of experiments conducted with the pig spleen enzyme, it appears that a more appropriate description of their function would be *protectors* rather than activators. A consideration of this property is contained in Section 3.4.7 below.

### 3.4.6 Inhibitors

Phosphofructokinase exists *in vivo* in a highly inhibited state. Suter (20), on the basis of his studies of glycolysis in rat spleen slices, estimated that even when glycolysis was occurring at its maximal rate (during anaerobiosis), phosphofructokinase activity only achieved about 10% of its maximal catalytic capacity, i.e. when assayed in the presence of optimal concentrations of substrates *in vitro*. In this section, the inhibitors identified in Section 3.4.4 are examined in greater detail, in an attempt to determine which molecules might be primarily responsible for the very low activity of phosphofructokinase *in vivo*.

MgATP is a potent inhibitor of pig spleen phosphofructokinase. In Figure 3-10 the ability of MgATP to inhibit the enzyme at two different concentrations of F6P is shown. Evidently the presence or absence of F6P is of considerable importance in determining the degree of inhibition by MgATP. In the presence of 0.2 mM F6P, the apparent $K_i$ for MgATP is only 1.3 mM, whilst in the presence of 0.5 mM F6P, the apparent $K_i$ increases to 3.0 mM. It is also noteworthy that in the presence of 0.5 mM F6P, maximal activity is not reached until 0.3 mM MgATP is present, whereas with only 0.2 mM F6P maximal activity is reached in the presence of only 0.1 mM MgATP. In the freeze-clamped rat spleen, the intracellular concentration of ATP is 4.3 mM, whilst that of F6P is only 0.05 mM (see Table 2-3). Clearly phosphofructokinase will be highly inhibited under these conditions.

In Figure 3-11 the inhibitory effects of citrate and PEP are shown. Citrate has a differential inhibitory effect on the enzyme from different

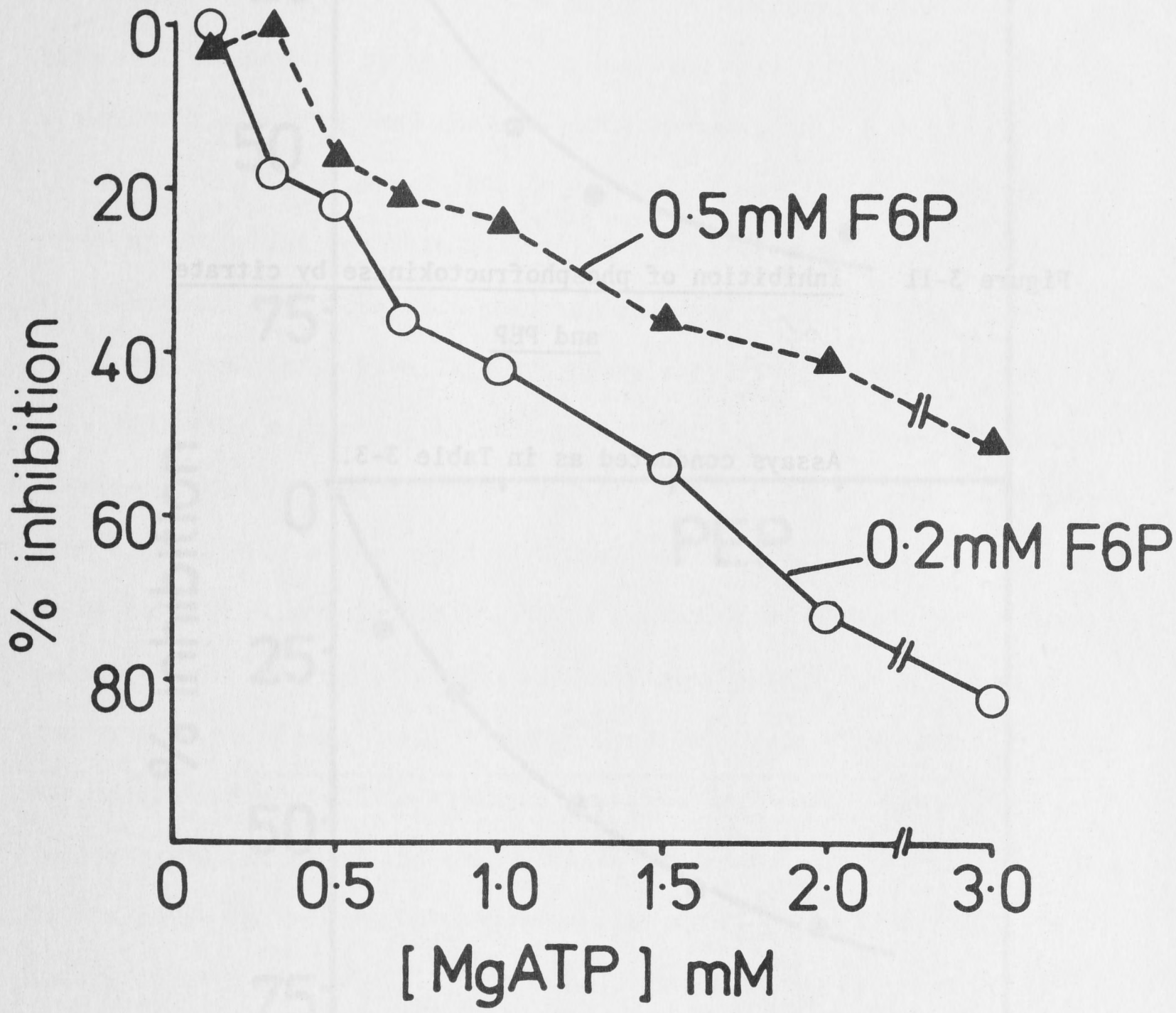
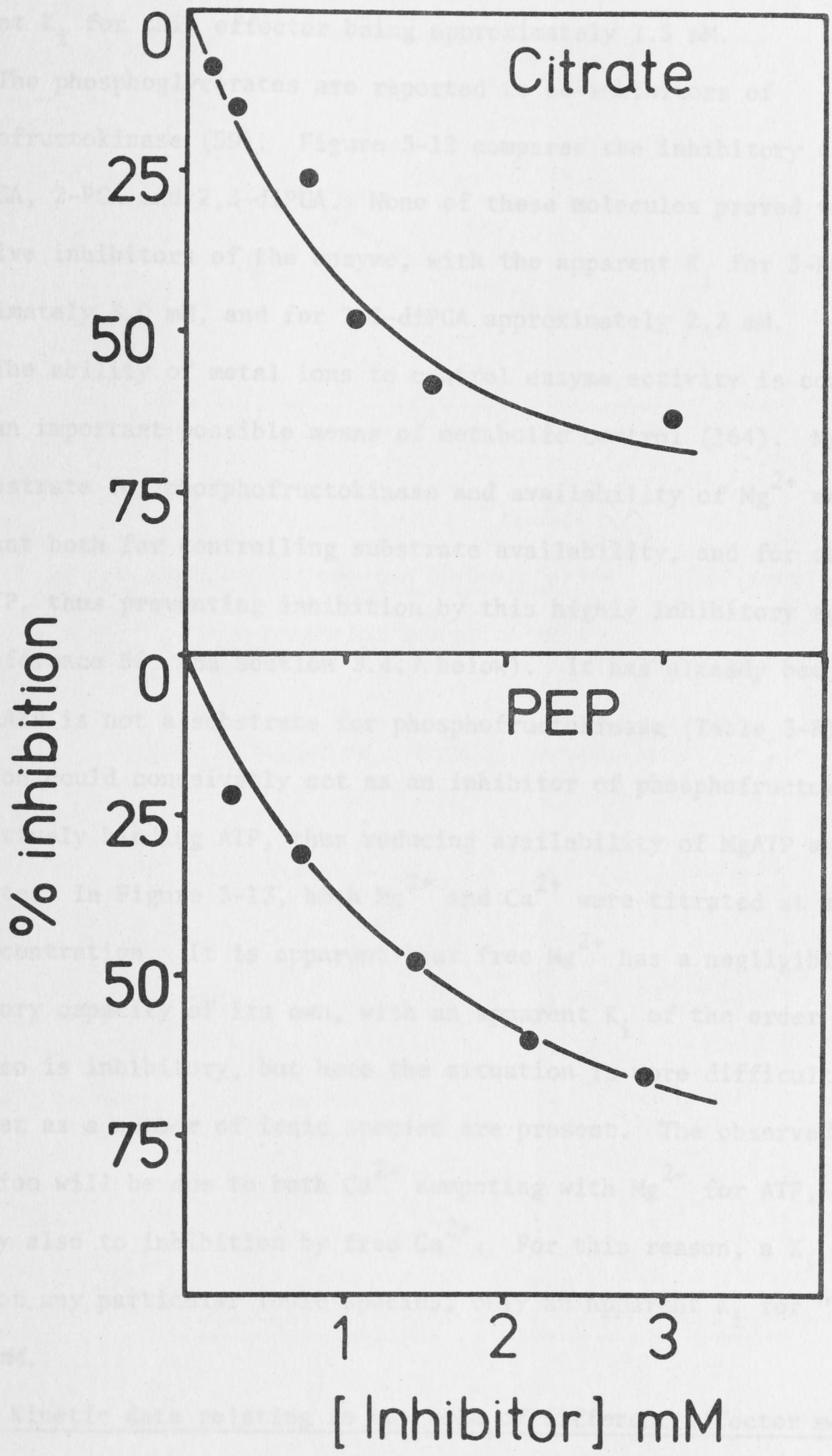



Figure 3-10 Inhibition of phosphofructokinase by MgATP, and the ability of F6P to counter this inhibition

Assays were conducted as in Table 3-3.

Figure 3-11 Inhibition of phosphofructokinase by citrate and PEP

Assays conducted as in Table 3-3.


Citrate
PEP
% inhibition
0
25
50
75
0
25
50
75
1
2
3
[ Inhibitor ] mM

sources, being a particularly potent inhibitor of muscle phosphofructokinase (83). In this study the apparent $K_i$ for citrate was found to be of the order of 1.2 mM. PEP had a very similar effect on enzyme activity, the apparent $K_i$ for this effector being approximately 1.5 mM.

The phosphoglycerates are reported to be inhibitors of phosphofructokinase (59). Figure 3-12 compares the inhibitory capacity of 3-PGA, 2-PGA and 2,3-diPGA. None of these molecules proved to be very effective inhibitors of the enzyme, with the apparent $K_i$ for 3-PGA being approximately 3.0 mM, and for 2,3-diPGA approximately 2.2 mM.

The ability of metal ions to control enzyme activity is considered to be an important possible means of metabolic control (164). MgATP is the substrate for phosphofructokinase and availability of $Mg^{2+}$ may be important both for controlling substrate availability, and for chelating free ATP, thus preventing inhibition by this highly inhibitory agent (see reference 58, and Section 3.4.7 below). It has already been shown that CaATP is not a substrate for phosphofructokinase (Table 3-8). This metal ion could conceivably act as an inhibitor of phosphofructokinase by competitively binding ATP, thus reducing availability of MgATP as a substrate. In Figure 3-13, both $Mg^{2+}$ and $Ca^{2+}$ were titrated at a fixed ATP concentration. It is apparent that free $Mg^{2+}$ has a negligible inhibitory capacity of its own, with an apparent $K_i$ of the order of 20 mM. $Ca^{2+}$ also is inhibitory, but here the situation is more difficult to interpret as a number of ionic species are present. The observed inhibition will be due to both $Ca^{2+}$ competing with $Mg^{2+}$ for ATP, and possibly also to inhibition by free $Ca^{2+}$. For this reason, a $K_i$ cannot be given for any particular ionic species, only an apparent $K_i$ for "calcium" of 1.2 mM.

### 3.4.7 Kinetic data relating to the role of different effector molecules

During the course of this work, it became apparent that the various

Figure 3-12 Inhibition of phosphofructokinase by phosphoglycerates

Assays conducted as in Table 3-3.

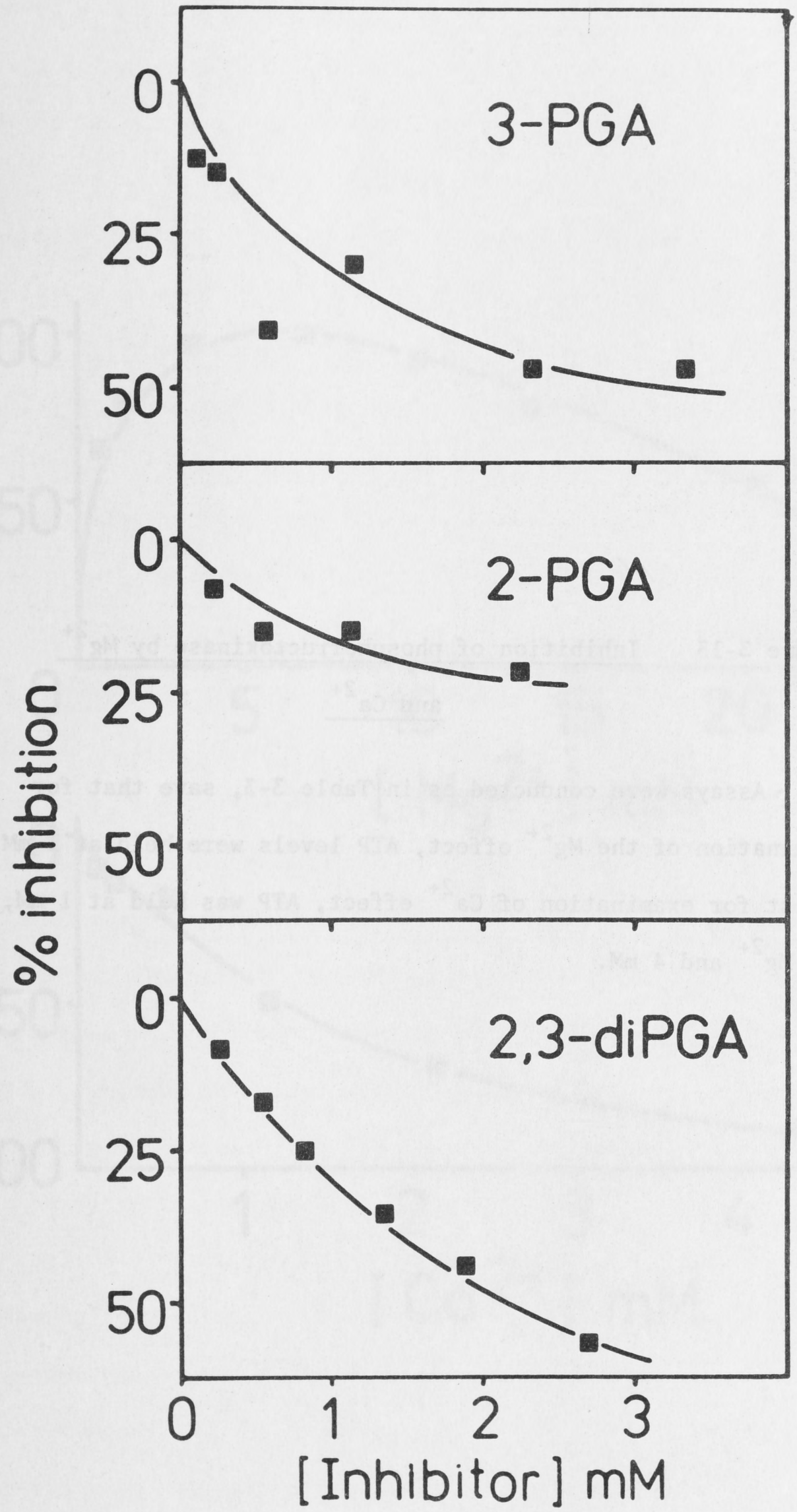
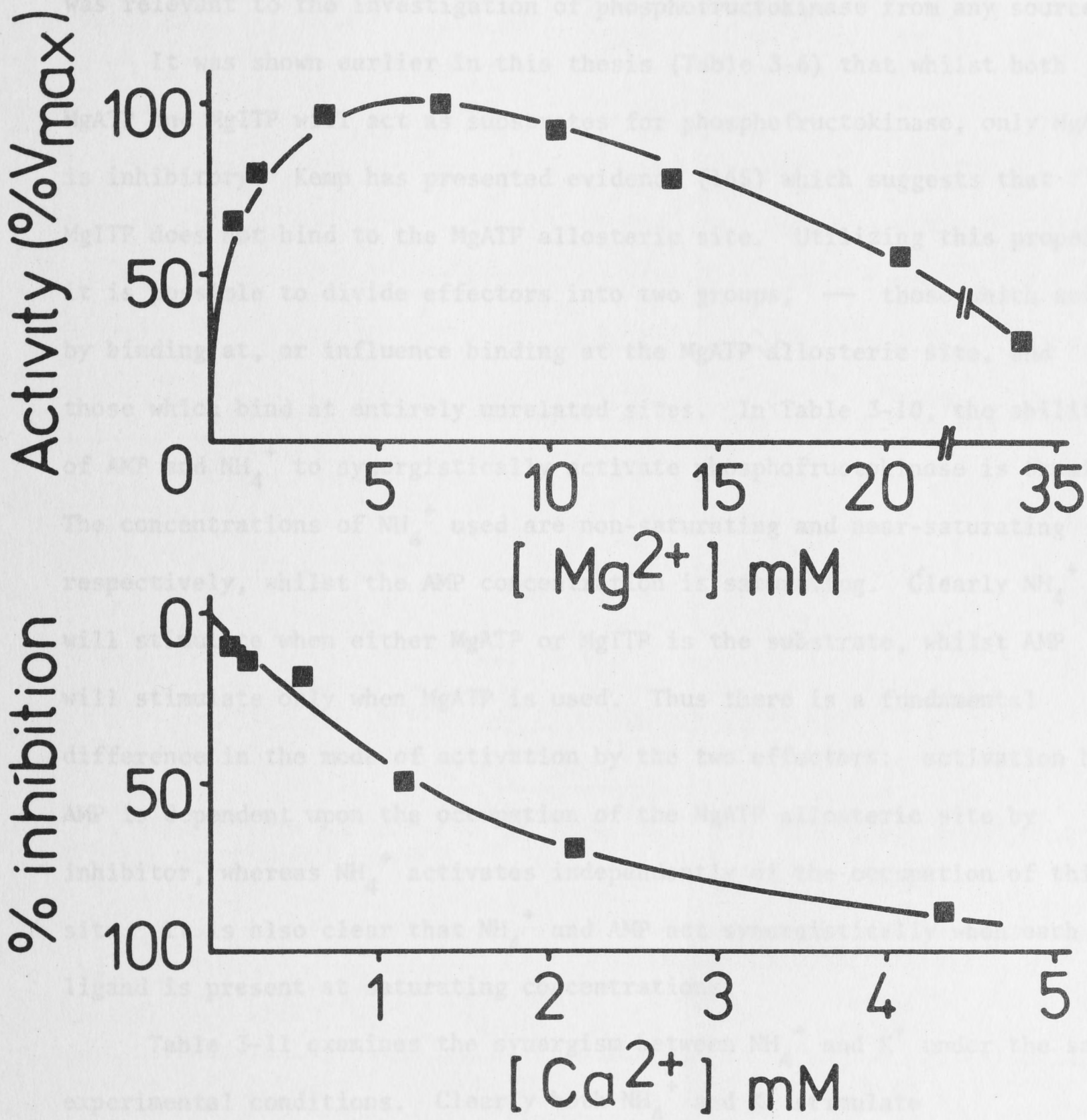

3-PGA
2-PGA
2,3-diPGA
0
25
50
% inhibition
0
1
2
3
[Inhibitor] mM

Figure 3-13 Inhibition of phosphofructokinase by $Mg^{2+}$ and $Ca^{2+}$

Assays were conducted as in Table 3-3, save that for examination of the $Mg^{2+}$ effect, ATP levels were held at 1 mM, whilst for examination of $Ca^{2+}$ effect, ATP was held at 1 mM, and $Mg^{2+}$ and 4 mM.


Activity (%Vmax)
100
50
0
5
10
15
20
35
[ Mg2+ ] mM
% inhibition
0
50
100
1
2
3
4
5
[ Ca2+ ] mM

effector molecules examined caused their alterations in enzyme activity in several different ways. In this section, kinetic evidence is presented which helps to clarify the *mechanism* by which various effectors modulate enzyme activity. Although it might be said that an investigation of the mechanism of control is not directly relevant to the mainstream of this thesis, it was felt that the information which could be obtained was relevant to the investigation of phosphofructokinase from any source.

It was shown earlier in this thesis (Table 3-6) that whilst both MgATP and MgITP will act as substrates for phosphofructokinase, only MgATP is inhibitory. Kemp has presented evidence (165) which suggests that MgITP does not bind to the MgATP allosteric site. Utilizing this property, it is possible to divide effectors into two groups, — those which act by binding at, or influence binding at the MgATP allosteric site, and those which bind at entirely unrelated sites. In Table 3-10, the ability of AMP and $NH_4^+$ to synergistically activate phosphofructokinase is examined. The concentrations of $NH_4^+$ used are non-saturating and near-saturating respectively, whilst the AMP concentration is saturating. Clearly $NH_4^+$ will stimulate when either MgATP or MgITP is the substrate, whilst AMP will stimulate only when MgATP is used. Thus there is a fundamental difference in the mode of activation by the two effectors: activation by AMP is dependent upon the occupation of the MgATP allosteric site by inhibitor, whereas $NH_4^+$ activates independently of the occupation of this site. It is also clear that $NH_4^+$ and AMP act synergistically when each ligand is present at saturating concentrations.

Table 3-11 examines the synergism between $NH_4^+$ and $K^+$ under the same experimental conditions. Clearly both $NH_4^+$ and $K^+$ stimulate phosphofructokinase activity when either MgATP or MgITP is used as the phosphate donor, and the activation by one ligand can be demonstrated in the presence of saturating concentrations of the other.

Table 3-10

Synergistic activation of phosphofructokinase by $NH_4^+$ and AMP

Assays were conducted as in Table 3-3, save that where appropriate, ITP was substituted for ATP.

| $NH_4^+$ (0.175 mM) | $NH_4^+$ (1.75 mM) | AMP (1.0 mM) | PHOSPHOFRUCTOKINASE ACTIVITY in MgATP (U/ml) | % stim. | in MgITP (U/ml) | % stim. |
|---|---|---|---|---|---|---|
| - | - | - | 0.256 | 0 | 0.246 | 0 |
| + | - | - | 0.569 | 120 | 0.610 | 150 |
| - | + | - | 1.003 | 290 | 0.819 | 230 |
| - | - | + | 0.453 | 90 | 0.273 | 10 |
| + | - | + | 0.919 | 260 | 0.567 | 120 |
| - | + | + | 1.367 | 430 | 0.831 | 240 |

Table 3-11

Synergistic activation of phosphofructokinase by $NH_4^+$ and $K^+$

Assays were conducted as in Table 3-3, save that where appropriate, ITP was substituted for ATP.

| $NH_4^+$ (10 mM) | $K^+$ (100 mM) | PHOSPHOFRUCTOKINASE ACTIVITY in MgATP (U/ml) | % stim. | in MgITP (U/ml) | % stim. |
|---|---|---|---|---|---|
| - | - | 0.31 | 0 | 0.26 | 0 |
| + | - | 1.47 | 374 | 1.61 | 619 |
| - | + | 2.37 | 663 | 2.26 | 871 |
| + | + | 3.61 | 1161 | 2.58 | 994 |

This result is important as it has been suggested that $NH_4^+$ and $K^+$ share the same binding site in analogous manner to pyruvate kinase (86). However, other workers have also observed stimulation by $NH_4^+$ in the presence of $K^+$ (83, 166). This result in no way conclusively proves that $NH_4^+$ and $K^+$ bind at separate sites, but it does suggest that $NH_4^+$ can stimulate phosphofructokinase *in vivo* in the presence of physiological concentrations of $K^+$.

As mentioned in Chapter 1, evidence has accumulated which suggests that MgATP binding to its allosteric site inhibits by causing the enzyme to dissociate into inactive subunits. The next group of experiments utilizes this property of rapid inhibition when phosphofructokinase is preincubated with MgATP. Preliminary experiments were conducted in which reactions were initiated by adding highly diluted phosphofructokinase to buffer (pH 6.70), which contained high concentrations of MgATP. This approach was dropped subsequently for two reasons:

(i) initiation of the reaction with enzyme, even under highly unfavourable circumstances, did not present as clear a picture of subsequent events as the method finally chosen; and

(ii) to visualize enzyme activity at all, a high concentration of $K^+$ had to be included in the assay buffer.

As will be shown later in this section, the presence of $K^+$ and F6P together can mask some very interesting results. A system where enzyme is preincubated with MgATP in the presence and absence of effectors, was finally chosen as the one from which most information could be obtained, particularly with respect to the mechanism by which effectors modulate enzyme activity.

Figure 3-14 shows the time course of inactivation of phosphofructokinase at two concentrations of MgATP, in the absence of other effectors. Reactions were initiated with 0.2 mM F6P and in all cases unless specifically mentioned, the reaction velocities were linear. This experiment shows clearly that the MgATP allosteric site is filled at very low concentrations of MgATP when F6P is absent (cf Figure 3-10), and that the inactivation process is a rapid one.

In Table 3-12, the effect of preincubating phosphofructokinase with a variety of molecules, both singly and together, is shown.

Table 3-12

The effect on activity of preincubating phosphofructokinase with various of its substrates

To cuvettes containing the indicated substrate(s), phosphofructokinase was added, then after 30 seconds the reaction was initiated with the other substrate(s). Samples marked "None", means that the reaction was initiated by adding phosphofructokinase to cuvettes already containing all the substrates.

| PREINCUBATION (30 seconds) WITH: | RELATIVE ACTIVITY |
|---|---|
| None (0.2 mM F6P) | 100 |
| Buffer alone | 111 |
| 0.4 mM ATP | 0 |
| 3.3 mM $Mg^{2+}$ | 0 |
| 0.2 mM F6P | 112 |
| 0.4 mM ATP + 3.3 mM $Mg^{2+}$ | 0 |
| 0.4 mM ATP + 0.2 mM F6P | 36 |
| 03.3 mM $Mg^{2+}$ + 0.2 mM F6P | 72 |
| None (1.0 mM F6P) | 113 |
| 1.0 mM F6P | 110 |
| 0.4 mM ATP + 1.0 mM F6P | 110 |
| 3.3 mM $Mg^{2+}$ + 1.0 mM F6P | 119 |

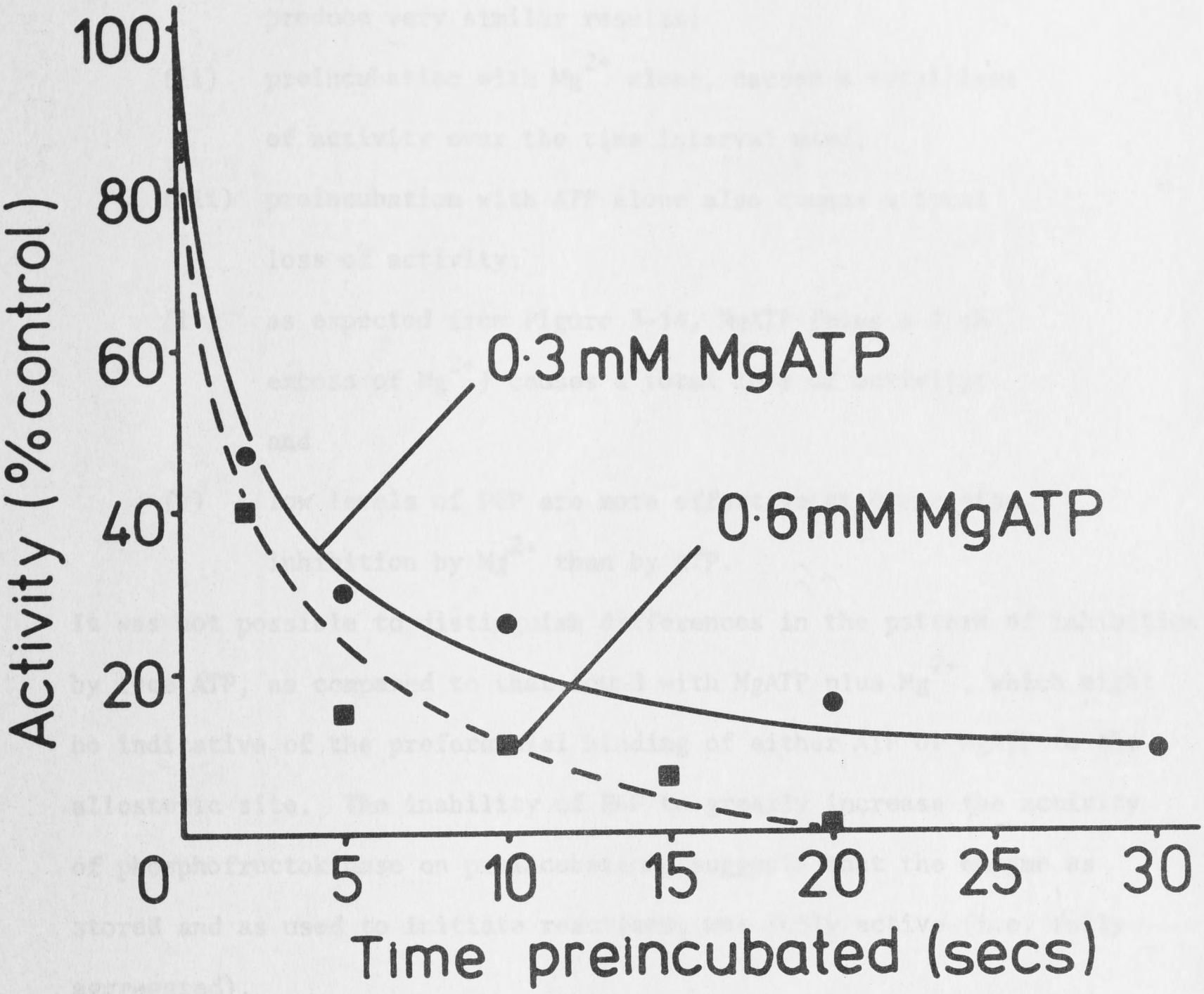



Figure 3-14 The effect on activity of preincubating phosphofructokinase with MgATP

To cuvettes containing the indicated concentration of MgATP, phosphofructokinase was added and after the appropriate time interval had elapsed, reactions were initiated with 0.2 mM F6P.

The following points are noteworthy:

(i) initiating the reaction with enzyme, and preincubating the enzyme either with buffer alone, or with F6P, produce very similar results;

(ii) preincubation with $Mg^{2+}$ alone, causes a total loss of activity over the time interval used;

(iii) preincubation with ATP alone also causes a total loss of activity;

(iv) as expected from Figure 3-14, MgATP (plus a 3 mM excess of $Mg^{2+}$) causes a total loss of activity; and

(v) low levels of F6P are more effective at overcoming inhibition by $Mg^{2+}$ than by ATP.

It was not possible to distinguish differences in the pattern of inhibition by free ATP, as compared to that found with MgATP plus $Mg^{2+}$, which might be indicative of the preferential binding of either ATP or MgATP to the allosteric site. The inability of F6P to greatly increase the activity of phosphofructokinase on preincubation, suggests that the enzyme as stored and as used to initiate reactions, was fully active (i.e. fully aggregated).

Figure 3-15 demonstrates the ability of F6P to block the inactivation of phosphofructokinase by ATP. If it is assumed that ATP inactivates the enzyme in similar fashion to MgATP, then this figure is of considerable interest when considering the *in vivo* situation, as it demonstrates how comparatively small changes in F6P concentration can change the activity of phosphofructokinase quite markedly.

In Table 3-13, data is presented which shows how various of the effector molecules influence the inactivation of phosphofructokinase, during preincubation with MgATP. At first glance there would only appear

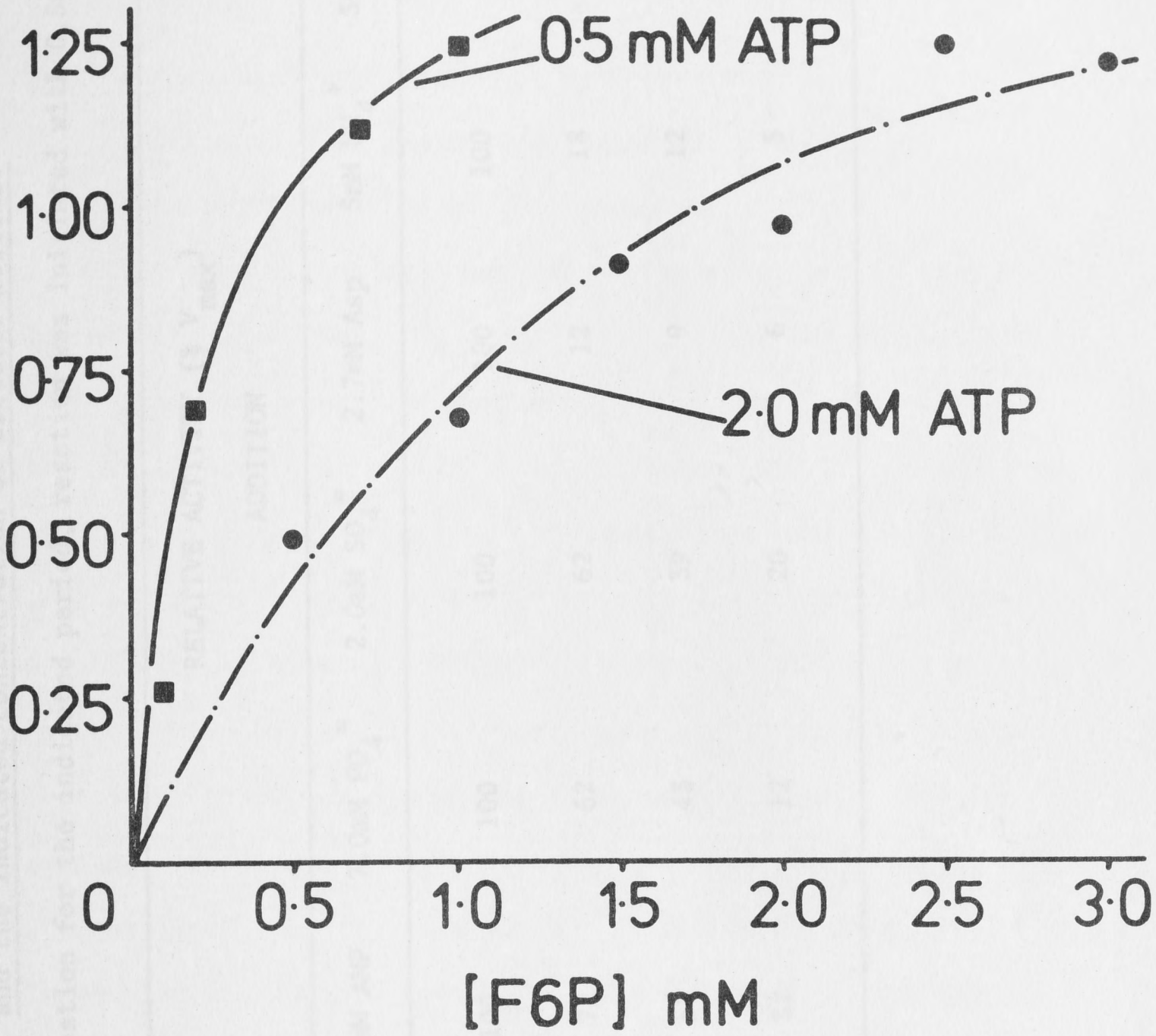



Figure 3-15 The ability of F6P to overcome the ATP preincubation of phosphofructokinase

Samples were preincubated with either 0.5 or 2.0 mM ATP, and the indicated concentration of F6P. After 20 seconds the reaction was initiated with either 3.3 or 5.1 mM $Mg^{2+}$.

Table 3.13 Time course of inactivation of phosphofructokinase when preincubated with 0.4mM Mg ATP and the indicated concentration of effector molecule

After preincubation for the indicated period, reaction was initiated with 0.5mM F6P.

| TIME PREINCUBATED (seconds) | RELATIVE ACTIVITY (% $V_{max}$) ADDITION | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NONE | 0.5mM AMP | 7.0mM $PO_4^=$ | 2.0mM $SO_4^=$ | 2.7mM Asp | 5mM $NH_4^+$ | 50mM $K^+$ | 1mM citrate |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 5 | 13 | 72 | 62 | 62 | 12 | 18 | 17 | 4 |
| 10 | 8 | 58 | 43 | 39 | 9 | 12 | 11 | 0 |
| 20 | 5 | 52 | 12 | 20 | 6 | 5 | 9 | 0 |

to be three groups of effector molecules present:

(i) those which significantly slow the inactivation process i.e. AMP, $PO_4^{2-}$ and $SO_4^{2-}$,

(ii) those which appear to have little effect when compared to the control, i.e. aspartate, $K^+$ and $NH_4^+$, and

(iii) those which accelerate the MgATP-mediated inactivation of phosphofructokinase. Citrate is a representative of this group.

However, further subdivision must be made of the effectors in groups (i) and (ii). Whilst all of AMP, $PO_4^{2-}$ and $SO_4^{2-}$ protect the enzyme, AMP is both an activator and protector, whilst $PO_4^{2-}$ and $SO_4^{2-}$ have only a protective function. In group (ii), with both $NH_4^+$ and $K^+$, on addition of F6P initial velocity rates were low, as indicated in Table 3-13. However, after a lag period of 10 - 15 seconds, velocity was observed to steadily increase till after 2 minutes a linear velocity was obtained, similar to that observed if the enzyme was not preincubated. It must be emphasized that in all other cases, when reaction rates were initiated with F6P reaction rates were *linear* with respect to time. It was difficult to quantitate the final velocity reached, but it did appear that the longer the preincubation the further from $V_o$ was the final velocity obtained. This might be taken to indicate that the inactivation of phosphofructokinase occurs in two stages, the first being a rapid inactivation which is readily reversible in the presence of appropriate effectors, and the second being a slower process which effectors are unable to reverse, at least over a short time interval.

Unfortunately, to determine the effect of FDP it would have been necessary to couple the phosphofructokinase reaction to pyruvate kinase and lactate dehydrogenase. Pyruvate kinase is active only in the presence

of $K^+$, and as shown above this ion produces an effect which would have masked the effect of FDP. However, as mentioned in Section 3.4.1, FDP was the most effective stabilizer of the stored enzyme. In this respect, its effect appears to be quite similar to that of $PO_4^{2-}$ and $SO_4^{2-}$.

The last part of this section deals with the role of enzyme concentration in determining both activity and kinetic behaviour. Figure 3-16 shows that enzyme activity is not linearly proportional to enzyme concentration. The earlier results in this thesis were obtained using a phosphofructokinase solution concentrated enough for activity to be directly proportional to concentration. To show this concentration-dependent effect it was necessary to go to a very low protein level (~300 µg/ml, prior to a further 20 - 100-fold dilution in the reaction cuvette). With phosphofructokinase as dilute as this, $K^+$ had to be added to ensure that activity could be visualized. The enzyme is saturated with $K^+$ *in vivo*, so the result here is very pertinent when considering the physiological role of phosphofructokinase. The activity using 50 µL of enzyme solution was nearly 20x greater than with 5 µL of enzyme, rather than the expected 10x greater activity.

Table 3-14 demonstrates that the cooperative behaviour of phosphofructokinase is also dependent upon enzyme concentration.

Table 3-14

The effect of dilution on the sigmoidal response of phosphofructokinase to F6P

Dilutions were conducted as in Figure 3-16. Assays were conducted as in Table 3-3.

| DILUTION FACTOR | HILL NO. |
|---|---|
| 1x | 1.24 |
| 2.5x | 1.65 |
| 5.0x | 1.87 |

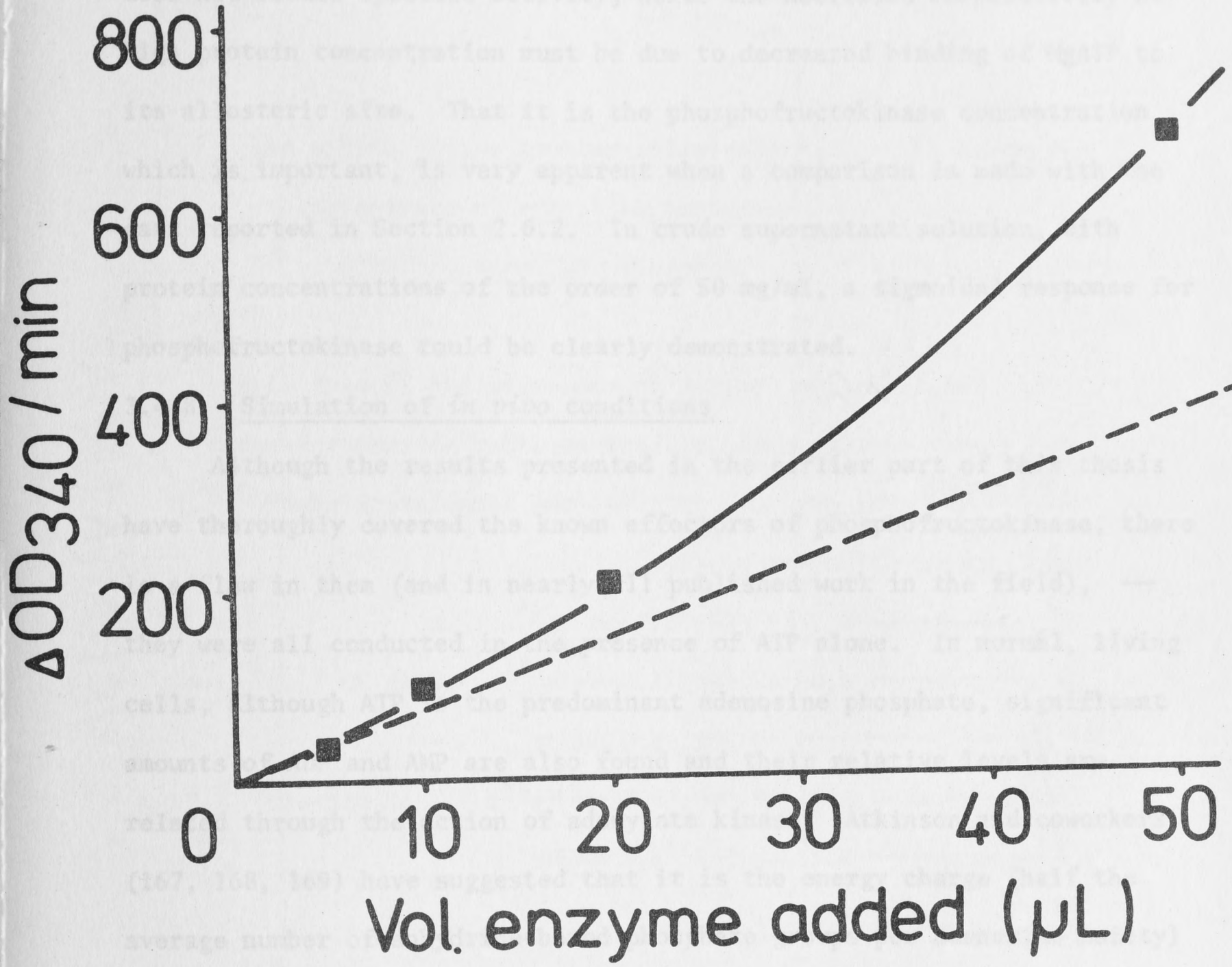



Figure 3-16 The effect of dilution on phosphofructokinase activity

Assays were conducted as in Table 3-3, save that 85 mM (saturating) $K^+$ was added. Stock phosphofructokinase solution was diluted in the same buffer (80 mM potassium phosphate + 1 mM EDTA, pH 8.0) till 5 μL enzyme gave a small but clearly readable OD change. This was a 12x dilution. To avoid discrepancies due to widely differing volumes of potassium phosphate buffer added (5 - 50 μL), enzyme + diluting buffer was always added to a total volume of 50 μL.

Ordinary stock solutions of phosphofructokinase gave a Hill Coefficient of only 1.24, whereas a 5x-diluted solution gave a value of 1.87. The value of the Hill Coefficient gives some indication of the extent of interaction between the MgATP allosteric and the F6P substrate sites. It is apparent from Figure 3-16 that increasing the concentration of enzyme does not reduce specific activity, hence the decreased cooperativity at high protein concentration must be due to decreased binding of MgATP to its allosteric site. That it is the phosphofructokinase concentration which is important, is very apparent when a comparison is made with the data reported in Section 2.6.2. In crude supernatant solution, with protein concentrations of the order of 50 mg/ml, a sigmoidal response for phosphofructokinase could be clearly demonstrated.

3.4.8 Simulation of *in vivo* conditions

Although the results presented in the earlier part of this thesis have thoroughly covered the known effectors of phosphofructokinase, there is a flaw in them (and in nearly all published work in the field), — they were all conducted in the presence of ATP alone. In normal, living cells, although ATP is the predominant adenosine phosphate, significant amounts of ADP and AMP are also found and their relative levels are related through the action of adenylate kinase. Atkinson and coworkers (167, 168, 169) have suggested that it is the energy charge (half the average number of anhydride-bound phosphate groups per adenosine moiety) which may be the primary determinant of phosphofructokinase activity.

In Figure 3-17 the role of the energy charge in regulating phosphofructokinase activity is examined. Rather than scan all the effector molecules that have been tested, representative effectors have been used. All activities are expressed relative to that observed at an energy charge of 0.59, under the conditions tested. Of particular interest is the extreme sensitivity to F6P concentration. In previous experiments done at an energy charge of 1.0, phosphofructokinase was strongly inhibited

Figure 3-17 The interaction between energy charge and effector molecules in regulating phosphofructokinase activity

Total adenine nucleotide concentration in each assay was 4 mM. Adenine nucleotides were added so that the ratio $\frac{[ATP][AMP]}{[ADP]^2}$ was as close to 0.44 (the adenylate kinase equilibrium value) as possible.

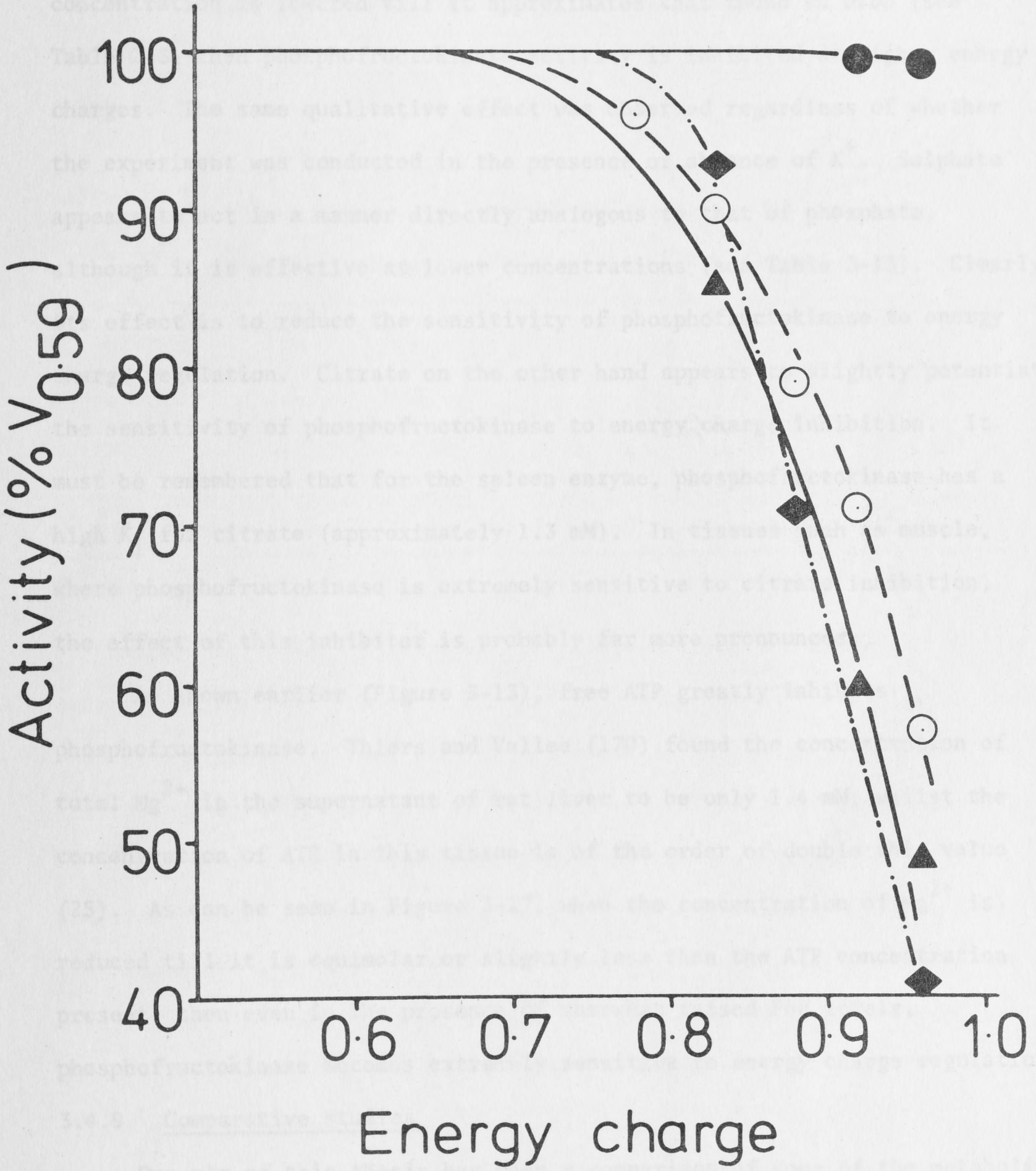
—●— 0.2 mM F6P, 5.5 mM $Mg^{2+}$.

—●— 0.06 mM F6P, 5.5 mM $Mg^{2+}$, 2.3 mM $SO_4^{2-}$.

- - -○- - - 0.06 mM F6P, 5.5 mM $Mg^{2+}$.

—▲— 0.06 mM F6P, 5.5 mM $Mg^{2+}$, 0.7 mM citrate.

—·—◆—·— 0.12 mM F6P, 3.3 mM $Mg^{2+}$.


100
90
80
70
60
50
40
Activity (% V0·59 )
0·6
0·7
0·8
0·9
1·0
Energy charge

in the presence of 0.2 mM F6P. In the presence of physiological ratios and concentrations of adenine nucleotides (energy charge ≃ 0.85), phosphofructokinase activity is completely unaffected when compared to the activity determined at a lower energy charge. If, however, the F6P concentration is lowered till it approximates that found *in vivo* (see Table 2-3) then phosphofructokinase activity is inhibited at higher energy charges. The same qualitative effect was observed regardless of whether the experiment was conducted in the presence or absence of $K^+$. Sulphate appears to act in a manner directly analogous to that of phosphate, although it is effective at lower concentrations (see Table 3-13). Clearly its effect is to reduce the sensitivity of phosphofructokinase to energy charge regulation. Citrate on the other hand appears to slightly potentiate the sensitivity of phosphofructokinase to energy charge inhibition. It must be remembered that for the spleen enzyme, phosphofructokinase has a high $K_i$ for citrate (approximately 1.3 mM). In tissues such as muscle, where phosphofructokinase is extremely sensitive to citrate inhibition, the effect of this inhibitor is probably far more pronounced.

As shown earlier (Figure 3-13), free ATP greatly inhibits phosphofructokinase. Thiers and Vallee (170) found the concentration of total $Mg^{2+}$ in the supernatant of rat liver to be only 1.4 mM, whilst the concentration of ATP in this tissue is of the order of double this value (25). As can be seen in Figure 3-17, when the concentration of $Mg^{2+}$ is reduced till it is equimolar or slightly less than the ATP concentration present, then even in the presence of somewhat raised F6P levels, phosphofructokinase becomes extremely sensitive to energy charge regulation.

### 3.4.9 Comparative studies

One aim of this thesis has been a comparison of some of the metabolic properties of spleen and thymus. In Table 3-15, some of the more important kinetic properties of pig spleen, rat spleen and rat thymus

phosphofructokinase are compared.

Table 3-15

Comparison of some apparent effector constants

for phosphofructokinase from various sources

The values for liver and muscle are from Kemp (54), and the values for pig spleen from earlier in this chapter.

| APPARENT EFFECTOR CONSTANT (mM) | PHOSPHOFRUCTOKINASE FROM: | | | | |
|---|---|---|---|---|---|
| | Liver | Muscle | Pig spleen | Rat spleen | Rat thymus |
| $K_i$ MgATP | 1.6 | 3.0 | 1.5 | 1.6 | 1.1 |
| $K_i$ citrate | >2.0 | 0.3 | 1.3 | 1.6 | —— |
| $K_A$ AMP | 0.21 | 0.035 | 0.03 | 0.03 | 0.04 |
| $K_A$ $NH_4^+$ | 0.35 | 0.35 | 0.35 | 0.37 | 0.40 |

For comparison, Kemp's corresponding values (54) for the liver and muscle enzyme are included. However, it must be emphasized that the enzymes he used were prepared in very different fashion to the lymphoid enzymes, and the various kinetic constants he obtained may not be genuinely comparable.

Pig spleen and rat spleen phosphofructokinases have essentially the same kinetic parameters. The only ones which are distinguishable are the respective $K_i$'s for citrate, and it is questionable whether these are significant. Of the three parameters measured for the thymus enzyme, the only difference distinguishing it from the spleen enzyme is that it is slightly more susceptible to MgATP inhibition. A more detailed consideration of the similarities and differences between spleen and thymus phosphofructokinase will be made in the Discussion. It is not possible

to say from the data above, whether the lymphoid enzyme is more similar to the liver or muscle enzyme. When compared to Kemp's data (54) the $K_A$ for AMP is almost identical with that of the muscle enzyme. Lymphoid phosphofructokinase is, however, far less sensitive to citrate inhibition than is the muscle enzyme. It appears likely from these studies that lymphoid phosphofructokinase is a hybrid form, and as suggested from electrophoretic data (171), intermediate between the liver and muscle enzymes in properties.

## 3.5 DISCUSSION

### 3.5.1 General considerations

Phosphofructokinase is an extremely labile enzyme, and considerable difficulty was experienced in developing a satisfactory purification method. The procedure of Massey and Deal (159) was finally adopted. One important point which must be considered here, is the significance of the low yield obtained. Such a result immediately raises the possibility of the specific loss of one or more isoenzymes. However, comparison of Massey and Deal's original data (159) with that in Table 3-1, shows that such an expectation is probably unreasonable. Liver phosphofructokinase is a homogeneous enzyme, and recoveries after successive purification steps were 39%, 36% and 17% (to the end of the "wash" step). Comparable recoveries in the present work were 67%, unknown and 21% (separate determinations were not conducted on the $Mg^{2+}$-precipitated enzyme). Further evidence that no losses were experienced during purification, can be found in the electrophoretic data of Kurata *et al* (171), who demonstrated the presence of two isoenzymes in crude extracts from rat spleen.

Although direct comparison with other results in the literature can frequently be misleading, the affinity of spleen phosphofructokinase for

DEAE-cellulose does bear some comment. Reported column fractionation methods have of course used different buffers and indeed, different materials (DEAE-cellulose or DEAE-Sephadex). The results however, have been qualitatively very similar: the muscle enzyme has a low affinity for DEAE-cellulose (or DEAE-Sephadex) and is eluted readily (56, 78), whilst the liver enzyme has a much greater affinity, and a considerable change in ionic strength is required before this isoenzyme is eluted (78). No reports have noted that any isoenzymatic form of phosphofructokinase did not bind to DEAE-cellulose or DEAE-Sephadex. It must be remembered that fractionation with DEAE-cellulose was tried by both column and batch preparative methods, the latter under identical conditions to those described by Tarui *et al* (57), and similar results were obtained each time. Two different batches of DEAE-cellulose were used at various times, and similar results were obtained. It must be concluded that at least one isoenzyme of spleen phosphofructokinase has somewhat different charge properties, to those of previously reported isoenzymes.

Studies on the pH optimum of spleen phosphofructokinase produced results essentially similar to those already recorded (56, 65). Under all three conditions tested, the pH optimum was found to be 8.2. As noted earlier, the different velocities observed at different pH's are very much a function of the relative concentrations of MgATP and F6P. At physiological pH, and in the presence of physiological concentrations of effectors, it is probable that phosphofructokinase will exist in a highly inhibited state. Intracellular pH can vary depending on the metabolic conditions that prevail, and it is probable, as Trivedi and Danforth have suggested (172), that small changes in the intracellular pH may be important in regulating the activity of phosphofructokinase.

The data presented in Figure 3-4 and its accompanying text, and Table 3-14, lend some credence to Bloxham and Lardy's suggestion (73) that

in some cases sigmoidal kinetics may represent an experimental artefact. Dilution of phosphofructokinase leads to inactivation (Figure 3-16) and to an increased sigmoidal response (Table 3-14). Binding of MgATP to its allosteric site is necessary for cooperative kinetics (102), and causes inactivation. Thus a sigmoidal response is a function of MgATP binding to its allosteric site. With little knowledge available as to the precise intracellular environment that phosphofructokinase exists in, it is not possible to offer any meaningful comment as to the importance or otherwise, that sigmoidal kinetics may play in the regulatory behaviour of this enzyme.

### 3.5.2 The effector molecules of spleen phosphofructokinase

The data contained in Tables 3-3 - 3-9 inclusive suggests that the following molecules may affect phosphofructokinase activity in some manner; ATP, ADP, AMP, cAMP, F6P, FDP, Aspartate, $K^+$, $NH_4^+$, $PO_4^{2-}$, citrate, and the interrelationship between divalent metal ions and ATP.

*Adenine nucleotides.* It has long been recognized that adenine nucleotides are the principal regulators of phosphofructokinase *in vivo* (89, 162). Both ATP and MgATP are potent inhibitors of enzyme activity (58, 67), whilst both ADP and AMP relieve this inhibition (162). Although considerable work has been done on the manner in which these individual effectors regulate phosphofructokinase activity, comparison of Figures 3-10 and 3-17, show that they are best considered together. Their role, under the heading of energy charge regulation of phosphofructokinase activity, will be discussed more fully in Section 3.5.4 below.

*F6P and FDP.* The role of F6P in regulating enzyme activity will also be considered in Section 3.5.4. The apparent lack of significant activation by FDP is the most puzzling result in this thesis. Lowry and Passoneau reported (58) that the apparent $K_A$ for this effector was of the order of 1 μM or less. In addition to conducting experiments directly on the effect of FDP using a pyruvate kinase/lactate dehydrogenase couple,

Figure 3-3 also supports the assertion that FDP is a weak activator. When the direct assay system was tested, FDP was allowed to accumulate. At the longest time interval used, FDP concentration in the cuvette was of the order of 20 µM, — well above the reported $K_A$, yet no suggestion of an increased rate was observed. On the other hand, FDP was found to be an excellent stabiliser of the enzyme. The net conclusion reached on the basis of the *in vitro* studies reported here, must be that FDP is an effector of phosphofructokinase, but that its effect on the spleen enzyme is far less pronounced than that observed for the enzyme from other sources.

*Aspartate.* During the preliminary screening of possible effector molecules, aspartate was found to activate the spleen enzyme with an apparent $K_A$ of approximately 0.7 mM. Greenbaum *et al* (88) calculated the cytoplasmic concentration of aspartate in liver to be 0.65 mM, and found that this level fell during starvation, and rose during the refeeding of a high carbohydrate diet. In addition to the finding that its level, *in vivo*, is very close to the apparent $K_A$, the excellent correlation between aspartate levels and phosphofructokinase activity, supports the contention that aspartate is a regulator of enzyme activity in liver.

The role of aspartate in regulating phosphofructokinase in spleen and thymus is less certain. Chan (120) has measured aspartate levels in both tissues, and calculated the cytoplasmic concentration by the method of Williamson (173). In spleen this value was 1.7 mM, and in thymus 2.5 mM, i.e. both well above the apparent $K_A$. Under no conditions observed, did the levels of aspartate drop to anywhere near the $K_A$ concentration. As however, the effects of other regulatory molecules can still be observed in the presence of aspartate, it is unlikely that aspartate is an important regulator in lymphoid tissue. It is more likely to be of importance in tissues like liver or muscle, where sensitivity to a wide range of effector molecules will enable large changes in activity to occur.

*$K^+$ and $NH_4^+$.* The strong activatory capacity of both $K^+$ and $NH_4^+$ has long been known (43, 45, 174). Although phosphofructokinase has a greater affinity for $NH_4^+$ (apparent $K_A$ 0.3 mM), than for $K^+$ (apparent $K_A$ 10 - 15 mM), $K^+$ appears to be favoured as it is present in the cell at concentrations which will saturate phosphofructokinase (84). $NH_4^+$ could still be an important regulatory molecule if there is a separate binding site for it on the enzyme. Several reports (52, 83, 166), in addition to work contained in this thesis, have reported activation by $NH_4^+$ in the presence of saturating concentrations of $K^+$. However, other work contained in this thesis has served only to emphasize the similarity of the activation by both ions. Final comment on the possible physiological role of $NH_4^+$ as an activator of phosphofructokinase, will have to await competitive binding experiments with homogeneous enzyme.

*Phosphate.* The results in this thesis are essentially in agreement with published reports (82, 175), that $PO_4^{2-}$ is an effector for phosphofructokinase. However, rather than the activation observed in earlier reports, the principal role of $PO_4^{2-}$ on the lymphoid enzyme appears to be as a protective agent. Further consideration of the role of $PO_4^{2-}$ is contained in Section 3.5.4.

*Citrate.* Citrate inhibits spleen phosphofructokinase as it does the enzyme from all mammalian tissues studied. Chan (120) has measured citrate levels in spleen and thymus, and found the cytoplasmic concentrations of both to be of the order 0.15 - 0.25 mM. Although citrate is undoubtedly a potent inhibitor of phosphofructokinase activity in tissues such as muscle, it appears unlikely that the spleen enzyme, which has a $K_i$ for citrate 5 - 10 times greater than the intracellular concentration, will be greatly affected.

*Divalent cations and free ATP.* Free $Mg^{2+}$ is a weak inhibitor, and it is unlikely that this ion would, itself, be of any importance as a

regulator of phosphofructokinase activity. Its more usual form within the cell is as a complex, either to various proteins, or as MgATP. $Ca^{2+}$ probably exists in very similar ionic forms as does $Mg^{2+}$. However, CaATP is not a substrate for phosphofructokinase (see Table 3-8), and thus may act as an inhibitor by competing with MgATP for the catalytic site. One intriguing possibility which remains, is that of inhibition of free ATP. Rolleston (176) has postulated that free ATP may be of physiological importance in regulating cerebral cortex hexokinase activity. As Lolley has shown (177), a large proportion of the divalent metal ions present within the cell, may be bound to intracellular components other than ATP. As demonstrated in Figure 3-17, when the levels of available $Mg^{2+}$ and ATP are finely balanced a slight increase in energy charge may cause a significant inhibition of enzyme activity by ATP. Thus the possibility that free ATP is a physiological regulator of phosphofructokinase activity cannot be dismissed lightly.

### 3.5.3 Mechanism of action of effector molecules

The introductory section and the results relating to phosphofructokinase have been consciously presented with the association/dissociation model for the enzyme in mind. So much evidence is available that phosphofructokinase exists in forms of different molecular weights (65, 68, 178, 179, 180, 181, 182) that further justification is not required.

With the information that is already available in the literature on the binding of effectors to phosphofructokinase, the data in this thesis provides a useful insight as to the mechanism of action of effector ligands. From the data collected in Section 3.4, it appears that:

(i) the binding of either ATP or MgATP to the allosteric site, inactivates the enzyme, probably by causing it to dissociate into inactive diners;

(ii) F6P, AMP and $PO_4^{2-}$ block this inactivation process, either by competitively binding to the ATP allosteric site, or by decreasing affinity at this site for (Mg)ATP;

(iii) citrate enhances the binding of ATP to its allosteric site;

(iv) aspartate appears to exert its stimulatory effect in some manner completely unrelated to the ATP allosteric site; and

(v) $K^+$ and $NH_4^+$ do not appear to *directly* influence binding to the ATP allosteric site, but appear to do so in the presence of F6P (see text associated with Table 3-13).

It must be emphasized that this data enables classification only into *groups*. Information as to the actual number and nature of binding sites, can only come with work on homogeneous enzymes. For this reason, no attempt is made to present a diagramatic model of phosphofructokinase.

### 3.5.4 Similarities of the spleen and thymus enzymes

As the data in this thesis has shown, spleen and thymus display similar metabolic properties, as do the phosphofructokinases from both sources. Although no work has been published comparing the kinetic properties of the spleen and thymus enzymes, two papers are relevant in this discussion. Yamada and Ohyama (63) partially purified the thymocyte enzyme and separated two isoenzymes by TEAE-cellulose chromatography, the minor one eluting in very similar fashion to a commercial muscle phosphofructokinase preparation. They compared some of the properties of the major component with those of the muscle enzyme, and found that the thymocyte enzyme was more susceptible to ATP inhibition, and more sensitive to activation by the other adenine nucleotides. Tsai and Kemp (81) tested

the effect of anti-sera to purified muscle and liver phosphofructokinases, on the enzyme from different tissues. Treatment with both anti-sera removed 98% of the activity of the spleen enzyme, but only 50% of the thymus enzyme activity. Thus, although similar in their kinetic behaviour, the spleen and thymus enzymes are *immunologically* distinct.

### 3.5.5 *In vivo* regulation of phosphofructokinase, in relation to the observed alterations in glycolytic flux

It has long been recognized that the adenine nucleotides together, are perhaps the primary regulators of phosphofructokinase activity (89, 162). It was Atkinson who first presented data (167) which showed that physiological concentrations of all the adenine nucleotides could cause profound changes in enzyme activity. Most studies on the regulation of phosphofructokinase activity have been conducted with ATP or AMP, either singly or together. ADP is known to be an activator, but its role has largely been ignored, even though its concentration is usually many times that of AMP. Kemp and Krebs have demonstrated (69) that ATP, ADP and AMP all bind competitively at the one allosteric site, so meaningful consideration of the *in vivo* regulation of phosphofructokinase activity, requires the role of all 3 nucleotides to be examined.

The data contained in Figure 3-17, suggests that F6P, free ATP and $PO_4^{2-}$ ($SO_4^{2-}$ acts in very similar manner to $PO_4^{2-}$) may all be important regulators of phosphofructokinase activity *in vivo*. Referring back to Chapter 2, four methods were used to perturb glycolytic flux. These were anaesthesia, anoxia, the GvH reaction and Con A stimulation. During the GvH reaction glycolytic flux rate appeared unchanged, whilst during the other conditions flux increased. Clearly regulation of phosphofructokinase is not by energy charge alone, as the only occasion on which this parameter changed largely was during anoxia, when it dropped in accordance with expectations. Under none of the conditions utilized,

was it possible to test for inhibition by free ATP. Only during the GvH reaction did both the energy charge and the ATP concentration increase, and even here the change was small with the energy charge going from 0.87 - 0.89. As the glycolytic rate does not change during the GvH reaction (20), no conclusions can be reached as to the possible role of free ATP in regulating phosphofructokinase activity.

Increased glycolytic flux correlates poorly with F6P levels, as during two of the three conditions which caused increased flux, F6P levels dropped. However, increased flux rates correlated very well with increases in both FDP and $PO_4^{2-}$. Although no value is shown for $PO_4^{2-}$ during anaesthesia, the large decrease in the adenine nucleotide pool ensured that $PO_4^{2-}$ levels increased. During the GvH reaction, when glycolytic flux appeared unchanged, FDP levels dropped, whilst $PO_4^{2-}$ levels remained constant. Thus the most effective correlation observed is for $PO_4^{2-}$.

Increased influxes of $PO_4^{2-}$ have been reported to occur during the early stages of mitogen stimulation of thymocytes (183). Reference to Figure 2-5 where the progressive activation of glycolysis in thymocytes is shown, provides further indirect evidence of a role for $PO_4^{2-}$ during the Con A-mediated stimulation of glycolysis. G6P is an inhibitor of hexokinase (33), and if elevated levels of this intermediate are to be maintained during glycolytic stimulation and glycogen synthesis, then the G6P inhibition must be overcome. $PO_4^{2-}$ relieves the G6P inhibition of hexokinase (33). Thus, this hypothesis perfectly fits the observed data for the Con A stimulation of glycolysis. During the GvH reaction however, there is some suggestion of an increased activity of the glucose carrier and/or hexokinase (as indicated by a decreased glucose concentration), in the presence of raised levels of G6P, and while $PO_4^{2-}$ levels remain constant. Thus the major problem outstanding in explaining glycolytic regulation in the lymphoid organs spleen and thymus, appears to be

associated with the other regulatory locus of the pathway, glucose transport/hexokinase.

It has not been the intention in this Section, to imply that $PO_4^{2-}$ is the sole regulator of phosphofructokinase activity. It has been demonstrated kinetically in this thesis, that many of the effectors of phosphofructokinase bind at different sites on the enzyme, and although their individual effects may be slight, it is quite conceivable that they might synergistically activate or inhibit the enzyme. However, both from *in vitro* experimentation and from examination of intermediate data, it does appear that $PO_4^{2-}$ plays a key role in regulating phosphofructokinase activity *in vivo*.

# CHAPTER 4

# PHOSPHOFRUCTOKINASE AND GLYCOLYSIS DURING LYMPHOCYTE TRANSFORMATION

# CHAPTER 4

# PHOSPHOFRUCTOKINASE AND GLYCOLYSIS DURING LYMPHOCYTE TRANSFORMATION

## 4.1 INTRODUCTION

This chapter is intended to provide a wider metabolic context for the work presented in Chapters 2 and 3, and in doing so to briefly consider work done at the whole cell level by others, in so far as it sheds light on the role of glycolysis during lymphocyte transformation.

## 4.2 PHOSPHOFRUCTOKINASE AND THE REGULATION OF GLYCOLYSIS IN LYMPHOID TISSUE

Chapters 2 and 3 have presented in considerable detail, evidence which shows that the regulation of glycolysis in lymphoid tissues, occurs primarily at two enzyme loci — the glucose carrier/hexokinase and phosphofructokinase. It is quite clear that two separate patterns of regulation can be distinguished:

(i) an *emergency glycolysis*, which functions when the tissue's respiratory system is challenged, and which depends upon the activation of phosphofructokinase as the primary metabolic response to the critically lowered energy charge associated with respiratory failure; and

(ii) *immunological glycolysis*, where phosphofructokinase can still be considered regulatory but where its activation appears to play a secondary role to the accelerated glucose transport and increased hexokinase activity.

A detailed examination of the effector molecules of phosphofructokinase, both as measured and as are reported in the literature, is consistent with the enzyme being regulatory under all of the conditions examined.

## 4.3 THE ROLE OF GLYCOLYSIS DURING LYMPHOCYTE ACTIVATION

The work contained in Chapters 2 and 3 fully covers the aims set out in the introduction to this thesis (see Section 1.6). This work would, however, be incomplete if some consideration were not given to the relationship it bears to the major problem: the specific role played by glycolysis during lymphocyte transformation. The work presented below is in no way intended to cover the entire topic, but rather to indicate in general terms, what is currently known about the activation of this pathway.

Recent evidence from this laboratory (121, 185) suggests that the stimulation of glycolysis, which occurs during the early stages of thymocyte activation by Con A, may be accompanied by the utilization of glucose by other pathways. One characteristic of *immunological glycolysis* is the increased steady-state concentration of hexose-monophosphates. G6P, besides being an intermediate in glucose oxidation, can also be diverted to glycogen synthesis and pentose phosphate formation, and it may be that accelerated flux through these pathways is of some importance for the transforming cell.

Accepting that there *is* a stimulation of glycolysis during lymphocyte transformation, the following possibilities appear to cover the likely explanations:

(i) enhanced energy production, in the form of ATP, is the major requirement that is fulfilled by accelerated conversion of glucose into lactate, in

cells that lack a large complement of mitochondria;

(ii) it is not ATP *per se*, but the increased concentration of G6P which is important. This intermediate also serves the pathways of glycogen and pentose phosphate synthesis, so elevation of its level places the cell in a state of *functional readiness* in the event of metabolic demands. Under these conditions, accelerated glycolysis may still be an essential part of the activation process, or merely a useful energy-yielding bonus.

(iii) The increased glucose uptake and metabolism may be a consequence of the membrane perturbations which accompany mitogen binding (186), leading to a specific influx of ionic signals (e.g. $Ca^{2+}$), which may lower cytoplasmic ATP levels, thus requiring an increment of ATP synthesis to make up this deficit via an increased glycolysis; or

(iv) the same membrane perturbations may lead to a non-specific stimulation of all carrier-mediated uptake processes, causing a gratuitous increase in glycolysis, due to increased intracellular glucose and phosphate levels.

### 4.3.1 Glycolysis as an energy source

In Table 4-1, oxygen consumption and glycolytic rates of rat thymocytes incubated in the presence and absence of Con A are presented, accompanied by calculations relating to the contribution to ATP synthesis under each condition. Weidemann and Kolbuch (121) have shown that 5 mM acetoacetate is readily oxidised by rat thymocytes and can contribute 66% to the fuel of respiration of the incubated cell suspension. Under these

Table 4-1

The stimulation of ATP synthesis during the first 3 hours of the Con A activation of thymocytes

The figures for the oxygen consumption and lactate production are from Weidemann and Kolbuch (121). In the absence of Con A, glucose contributes 37% to the oxygen consumption, whilst in the presence of Con A this value is 45% (calculated on the basis of dilution of label in $^{14}CO_2$). It is assumed that the balance of the oxygen consumption comes from triglyceride. Calculations are based on a P/O ratio for pyruvate of 3 and a P/O ratio for triglyceride-derived fatty acids of 2.7.

| | - Con A | + Con A |
|---|---|---|
| oxygen consumption ($\mu$ moles/$10^{10}$ cells/3 hr.) | 360 | 391 |
| glycolytic rate ($\mu$ moles glucose equiv./$10^{10}$ cells/3 hr.) | 33 | 96 |

| | ATP synthesis ($\mu$ moles/$10^{10}$ cells/3 hr.) | |
|---|---|---|
| oxidative | 2024 | 2216 |
| glycolytic | 66 | 192 |
| TOTAL | 2090 | 2408 |

conditions, where acetyl-CoA enters the Krebs cycle independently of pyruvate dehydrogenase, neither the oxygen consumption nor the utilization of acetoacetate by the cells is stimulated by Con A. Conversely, the presence of acetoacetate together with glucose does not diminish the accelerated conversion of glucose to lactate, brought about by Con A treatment. Thus the stimulation of oxygen consumption in the presence of Con A, when glucose is the added substrate, is dependent on the activation of a site on the pathway of glucose oxidation prior to the oxidation of acetyl-CoA, and can be explained on the basis of the 3 - 4 fold increase in the pyruvate concentration (10 to 40 μM) which accompanies the rise in lactate formation. Thus the entire 15% increase in ATP synthesis is ultimately dependent upon the stimulation of the glycolytic rate.

4.3.2 Glucose utilization for biosynthetic events

Table 4-2 presents in summary form the major parameters measured during examination of [U-$^{14}$C]-glucose oxidation by Con A-stimulated thymocytes. Between 20 and 40% of the glucose carbon utilized is unaccounted for by the products measured, and although a small proportion of the label may be incorporated into intermediates like glutamate and aspartate by exchange reactions (20) without net synthesis, the majority of the unidentified carbon may be metabolized via pathways other than glycolysis. Puckle (185) has shown for example, that the incorporation of label from [U-$^{14}$C]-glucose into the protein and RNA fractions isolated from rat thymocytes more than doubles during the first 3 hours of Con A-stimulation. The implication is that the elevated levels of G6P are connected with the utilization of glucose carbon for purposes other than merely ATP synthesis. During the later stages of immunological reactions (e.g. the GvH reaction and long term studies on mitogen activation) the rate of nucleic acid and protein synthesis increases markedly (20, 118). Weidemann has suggested (121) that a small, but perhaps still significant

Table 4-2

Stimulation of glycolysis in rat thymocytes

This data is from Weidemann and Kolbuch (121).

| | GLUCOSE ALONE | CON A | A23187 |
|---|---|---|---|
| | ($\mu$ moles/$10^{10}$ cells/3 hr.) | | |
| OXYGEN CONSUMPTION | 360 | 391 | 362 |
| GLUCOSE DISAPPEARANCE | 55 | 122 | 117 |
| LACTATE PRODUCTION | 27 | 134 | 98 |
| PYRUVATE PRODUCTION | 1.3 | 4.5 | 6.2 |
| LACTATE/PYRUVATE | 20.7 | 29.7 | 16 |
| $^{14}CO_2$ PRODUCTION | 19 | 26 | 31 |
| GLUCOSE NOT ACCOUNTED FOR | 21.5 | 26.5 | — |

portion of the glucose utilized and unaccounted for, may be either stored initially as glycogen, or utilized directly via the pentose phosphate pathway as a net source of ribose moieties for nucleic acid synthesis.

4.3.3 Specific activation by $Ca^{2+}$

A23187 is a carboxylic acid ionophorous antibiotic which is specific for divalent metal ions (187). It forms lipid soluble complexes with cations, thereby facilitating diffusion of the cation across biological membranes. Maino *et al* (188) have recently used this ionophore to demonstrate that $Ca^{2+}$ influx can initiate transformation of pig lymph node lymphocytes, as judged by the uptake of [6-$^{3}$H]-thymidine into DNA after 45 hours of culture, and by morphological changes characteristic of lymphoblast formation.

Table 4-2 presents data showing the effects on glucose metabolism of incubating rat thymocytes with A23187 in a normal phosphate buffered saline containing 1.2 mM $CaCl_2$. The ionophore produces early metabolic changes which are qualitatively very similar to those observed with Con A; stimulating glucose uptake, lactate production and glucose oxidation. There is, however, no stimulation of oxygen consumption, no increased incorporation of labelled glucose into the protein and RNA fractions and, as judged by the lactate/pyruvate ratio, the cytoplasmic $NAD^{+}$/NADH ratio is more oxidised than with mitogen. Furthermore, the Con A stimulation is partially sensitive to puromycin inhibition, whilst the A23187 stimulation is not (121). There is an absolute requirement for $Ca^{2+}$ ions for transformation by the mitogen phytohaemagglutinin (189), which rapidly stimulates $Ca^{2+}$ uptake (111). Although it may appear naive to suggest that $Ca^{2+}$ *alone* is responsible for initiating transformation, the experimental evidence supports the view that accelerated entry of this cation may play a key role in the process. Weidemann has recently demonstrated (121), using the $Ca^{2+}$-buffer nitrolo-triacetate (190), that

increasing the cytoplasmic free $Ca^{2+}$ concentration from 20 μM to 40 μM is sufficient to cause the stimulation of glucose uptake shown in Table 4-2. The mechanism of this stimulus is unknown, but considering that an increase in the cytoplasmic free $Ca^{2+}$ concentration would remove the inhibition which normally prevents the translocation of ATP from the cytoplasm back into the mitochondria (191), a strong possibility exists that deinhibition of both hexokinase and phosphofructokinase, occurs as a consequence of the removal of ATP from the cytoplasm.

4.3.4 Non-specific glycolytic stimulation

The first event which occurs during lymphocyte transformation, is the binding of the mitogen to glycoprotein receptors on the outer surface of the lymphocyte's plasma membrane. Immediately after this binding, a whole series of membrane changes occur. Resch *et al* (192) have demonstrated a stimulated incorporation of oleate into membrane lecithin immediately after the surface binding of phytohaemagglutinin, whilst Fisher and Mueller (109) have similarly shown a stimulated incorporation of $^{32}PO_4^{2-}$ into phosphatidic acid. Singer and Nicholson (193) have shown that changes in the membrane phospholipids can cause perturbations in the membrane proteins, whilst at the same time there is a greatly increased fluidity of membrane lipids (186). When it is considered that these processes correlate with permeability changes to molecules as diverse as glucose (194), $K^+$ (194), $Ca^{2+}$ (111), hexoses (112), γ-aminobutyric acid (113), phosphate (183) and uridine (114), then it must be admitted that the membrane changes may be so great as to cause a general activation of carriers.

4.3.5 Conclusions

Of the four possibilities presented above, there is no evidence available at present which demonstrates any one to be correct at the expense of any other. However, certain conclusions can be reached.

The involvement of $Ca^{2+}$ in the transformation of lymphocytes is indisputable:

(i) its presence is essential for mitogen transformation (189);

(ii) mitogens enhance its uptake by the lymphocyte (111); and

(iii) $Ca^{2+}$ alone can induce transformation in the presence of an appropriate ionophore (188).

It is conceivable that the stimulated uptake of $Ca^{2+}$ is simply part of the generalized carrier activation already considered. We do not believe this to be likely, however, for several reasons.

On purely teleological grounds, this hypothesis is unacceptable. Transformation converts the lymphocyte from a comparatively quiescent state to one of intense metabolic activity. The synthesis of nucleic acids, proteins and other complex cellular components required before mitosis can occur, necessitates the utilization of considerable energy, and it is highly unlikely that energetically-unnecessary steps such as the transport of non-essential metabolites would occur.

The experiments reported in Section 4.3.3 above, could be considered as indirect evidence for this argument. Entry into the cell of only one ion, $Ca^{2+}$, causes transformation. This is specificity of the highest order. Recent work by Crumpton and coworkers (M.J. Crumpton, personal communication) suggests that entry into the cell of $Ca^{2+}$ alone (using A23187) initiates a sequence of metabolic events similar to that which occurs after the binding of mitogen. This strongly suggests that the membrane-regulated fluxes of ions and metabolites into the lymphocyte are *dependent upon the prior entry* into the cell of $Ca^{2+}$, and as this proposed initial step is highly specific, it is perhaps reasonable to believe that the associated fluxes are also specific. It is with this hypothesis in

mind that two possible explanations for the observed glycolytic stimulation will now be considered.

As stated earlier, the binding of Con A to the lymphocyte plasma membrane initiates a whole sequence of biosynthetic events which continue for several days and require a considerable expenditure of energy. Lymphocytes have little glycogen or triglyceride stored for use as an energy-providing fuel (20) so it is entirely possible that glycolysis is essential to enable the required synthetic activity to occur. Under these conditions, stimulated transport of glucose and phosphate for example, could be considered as specific events in that they accelerate an essential associated process. In Section 4.3.1 above, it was calculated that the stimulation of glycolysis increased ATP synthesis by 15%, and considering that glycolytic stimulation is still continuing after 3 days (184), the increment of ATP gained from glycolytic stimulation is considerable. Weidemann has suggested (M.J. Weidemann, personal communication) that the observed oxygen consumption during Con A stimulation, is approaching the maximal capacity of the lymphocyte's respiratory system. Such a maximal utilization of the cell's energy-producing resources, would support the contention that a maximal return from the cell's energy-producing system is essential for lymphocyte transformation.

The other possible explanation for the observed glycolytic stimulation is also consistent with glycolysis being essential in the transformation process, but for a different reason. Raising the intracellular $Ca^{2+}$ concentration may cause both an uncoupling of oxidative phosphorylation, and an uptake of ATP by the mitochondria (191). The net effect would be a lowered cytoplasmic ATP concentration, and this would lead to a stimulated glycolytic rate, as a consequence of deinhibition of phosphofructokinase and perhaps also of hexokinase. The slightly different

emphasis here is that glycolysis is necessary to repair the metabolic "damage" done by $Ca^{2+}$, rather than to promote biosynthetic activity.

No consideration has been given as yet in this section, to the possibility of glucose carbon being utilized via pathways other than glycolysis. Lymphocytes have little glycogen stored for use as an energy-providing fuel in time of need, and it is certainly conceivable that in the early stages of lymphocyte activation, when the energy demand is relatively light, carbon may be diverted into glycogen for use at a later stage of the transformation process. No evidence is available to support this hypothesis and it is presented merely as a possibility. The pentose phosphate pathway provides, amongst other things, pentose moieties for nucleic acid synthesis. Suter (20) has shown a large stimulation of incorporation of glucose carbon into nucleic acids 5 days after the initiation of the GvH reaction. It is not possible to say at present whether glucose carbon is of any importance in nucleic acid synthesis under these conditions.

In summary: the primary cytoplasmic event in the transformation of lymphocytes appears to be an elevation of the intracellular $Ca^{2+}$ concentration. Following this entry of $Ca^{2+}$, a series of membrane-associated events occur which lead to a stimulated influx into the cell of a variety of ions and metabolites. Shortly thereafter, glycolytic rate increases, whether as a consequence of stimulation by elevated levels of $PO_4^{2-}$, or as an indirect stimulation, by $Ca^{2+}$ causing decreased cytoplasmic ATP concentrations, is uncertain. Either or both of these will coordinately increase the activity of both hexokinase and phosphofructokinase. The glycolytic stimulation continues for at least 3 days, and it is estimated that this stimulus leads to an increase in ATP synthesis of the order of 15%. In view of the great demand for energy during the transformation process, glycolysis may well be essential as an energy source.

## 4.4 FUTURE WORK

It is clear from the work presented in the previous section, that the dominant question associated with lymphocyte transformation at the present time is the precise nature of the transforming agent. Although Maino *et al* (188) found that $Ca^{2+}$ (with A23187) would initiate transformation in a qualitatively very similar manner to that observed with mitogen, the degree of stimulation, as judged by tritiated thymidine incorporation into DNA, was only about one-third that obtained when phytohaemagglutinin was the stimulating agent. This may indicate that only a sub-population of the mixed T and B cells of a pig lymph node is susceptible to transformation by $Ca^{2+}$. The possibility of a cotransforming or potentiating agent which would increase the sensitivity of the cells to $Ca^{2+}$ is also very real. Allied to this problem is the need for an in-depth investigation of the multitude of events which both accompany and follow mitogen binding and, in particular, a description of their precise sequence and mutual dependency. The questions to be asked here are:

(i) are the membrane-associated and intracellular metabolic events which follow mitogen binding all *essential* for the transformation process?; and, if so,

(ii) what is their specific function?

It is still not proven that glycolytic activation is *essential* for lymphocyte transformation, even though the energy increment generated by this process is considerable.

Although a great deal of exploratory work still remains to be done at the whole cell level, this thesis was written primarily in the context of regulation at the enzyme level, and some consideration must be given to future work in this direction.

Regulation of glycolysis in lymphoid tissue occurs at two distinct loci: glucose transport/hexokinase, and phosphofructokinase. Evidence available suggests that the primary regulatory events encountered during immunological stimulation occur at the sites of glucose entry and/or phosphorylation. No detailed kinetic investigations of either the glucose carrier or hexokinase have been reported for lymphoid tissues. In view of the hypothesis proposed in this thesis, i.e. that increased levels of intracellular phosphate may coordinately activate both hexokinase and phosphofructokinase during transformation, a detailed examination of the kinetic and allosteric properties of a lymphoid hexokinase needs to be undertaken.

Recent evidence from Laird and Weidemann (personal communication) indicates that glucose transport occurs in unstimulated rat thymocytes at a very low initial rate, consistent with rate-limitation of carbon flux at the point of glucose entry. Questions to be answered in relation to the glucose carrier include:

(i) does the *rate* of transport change on mitogen binding, or do the membrane perturbations described earlier uncover cryptic carrier sites, thus increasing the maximal velocity for transport, by increasing the total number of effective carrier molecules available?; and

(ii) does reverse transport play an important role in determining the intracellular concentration of glucose, and if so, do the characteristics of this reverse transport process change after mitogen binding?

Once the mechanism of regulation at these two inter-related sites is more clearly understood, further efforts will need to be directed to

understanding the inter-relationship of glucose metabolism via glycolysis, with the regulation of related pathways e.g. the pentose phosphate pathway, and ultimately, to the whole pattern of energy-producing and precursor-generating metabolic processes, both cytoplasmic and mitochondrial, associated with lymphocyte transformation.

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# BIBLIOGRAPHY


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# PHOSPHOFRUCTOKINASE AND THE REGULATION OF GLYCOLYSIS IN LYMPHOID TISSUE

## ERRATA

1. The following abbreviations should be included on page v.

   adrenocorticotropic hormone ..... ACTH

   ATP:D-fructose-6-phosphate 1-phosphotransferase (E.C. 2.7.1.11) .... phosphofructokinase

   sodium dodecylsulphate .... SDS

2. On p. 22 the $K_A$ for $PO_4^{2-}$ is quoted as being "of the order 4-5 mM", but no reference is given. The appropriate reference is (58), but should be accompanied by a statement that the apparent $K_A$ is dependent upon both the ATP and F6P concentrations.

3. On p. 39 should be 'inulin-($^{14}$C-carboxylic acid)', and on the second-last line, 'temperature' is spelt incorrectly.

4. On p. 49, para. 3, line 2, 'intravascular' is incorrectly spelt.

5. On p. 78, the units for ATP/ADP x $P_i$ are $M^{-1}$.

6. On p. 82, the Hill coefficient is referred to without a reference; this is Hill A.V. (1910) J. Physiol. 40, 4P.

7. On p. 84, four lines from the top of the page, should read '....to that in 10 mM pyruvate'.

8. On p. 88, eight lines from the top of the page, 'thymi' is incorrectly spelt.

9. On p. 91, last paragraph, it should be stated that all enzyme assays were conducted at 23°.

10. On p. 110, no explanation is given of the ratio $\frac{V_L}{V_H}$ in Table 3.6. The legend should explain that this ratio is a comparison of the enzyme's activity in 0.3 mM NTP as opposed to that in 3.0 mM NTP.

ERRATA (continued)

11. Legend to Fig. 3.13, p.126, last line should read 'and $Mg^{2+}$ at 4 mM'.

12. On p. 135, half-way down page, is the statement 'reaction rates were initiated with F6P'. Delete 'rates'.

13. On p. 137, in the legend to Figure 3-16, should be included a statement to the effect that the dashed line represents the expected curve if the activity is proportional to enzyme concentration.

14. On p. 146, seven lines from the top is found 'Several reports (52, 83, 166), ....'. Reference 52 should not be included here as these workers used $(NH_4)_2.SO_4$.

15. On p. 147, the last word should be 'dimers'.

16. On p. 157, Table 4.2, with A23187 'glucose not accounted for' should be 23.9.

ERRATA (continued)

BIBLIOGRAPHY

Reference 4. 'Robison A.' should read 'Robison R.'

Reference 21. 'Eppenberger R.' should read 'Eppenberger H.'

Reference 23. 'Koven B.' should read 'Koven B.J.'

References 27,58,74,87,92,154 and 162. 'Passoneau J.V.' should read 'Passonneau J.V.'

Reference 33. 'p.285' should read 'p.277'

Reference 59. 'Matchinsky F.M.' should read 'Matschinsky F.M.'

Reference 73. '(1972)' should read '(1973)'

Reference 89. 'p. 584' should be inserted.

Reference 97. 'Holland P.G.' should read 'Holland P.C.'

References 101 and 116. Journal should be abbreviated 'Hoppe-Seyler's Z. Physiol. Chemie'.

Reference 148. 'Singh R.' should read 'Singh R.M.M.'

Reference 151. Page number is '2965'.

Reference 153. 'Raijmah L.' should read 'Raijman L.'

Reference 156. '*Energy Levels*' should read *'The Energy Level'*

Reference 177. 'Lolley R.M.' should read 'Lolley R.N.'

Reference 189. 'Ahlford' should read 'Alford'.